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Horticultural potential and population genetics of Nyssa aquatica L. (water tupelo)

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Horticultural potential and population genetics of *Nyssa aquatica* L. (water tupelo)

by

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ABSTRACT

Trees native to seasonally flooded wetlands have proven valuable in managed landscapes where wide fluctuations in soil moisture occur regularly. *Nyssa aquatica* L. (water tupelo) is a large, deciduous tree native to continuously and periodically flooded wetlands of the southeastern United States. I investigated the potential for using this tree in horticulture based on its tolerance to extreme fluctuations in soil moisture, water deficit, and its capability to be propagated asexually via stem cuttings. I also examined the genetic structure of the species across its native range by using inter-simple sequence repeats (ISSRs). Seedlings of *N. aquatica* were more tolerant to water deficit than seedlings of *Nyssa ogeche* Bartram ex. Marsh. (Ogeechee tupelo), another native North American species indigenous to wetland soils. Up to 40% mortality was observed for *N. ogeche* when subjected to repeated cycles of soil drying, whereas a maximum of 20% mortality was observed for *N. aquatica* subjected to the same treatment. However, up to 55% of *N. aquatica* plants died when subjected to continuous water deficit. Seedlings of *N. aquatica* were tolerant to wide fluctuations in soil moisture but were sensitive to long-term water deficit when growth potential was high during spring and early summer. Plants exposed to moderate soil moisture before water deficit exhibited reduced photosynthetic rates and predawn water potentials, whereas plants subjected to a water deficit pretreatment or flooding before water deficit had photosynthetic rates and predawn water potentials similar to those of well-watered controls. Seedlings treated with flooding before being exposed to water deficit also maintained greater aesthetic appeal than those pretreated with moderate soil moisture. Slower growth rates and reduced leaf area resulted when plants were treated with water deficit or flooding, which led to reduced transpirational demand and probably

contributed to increased tolerance to future water deficit compared with plants pretreated with moderate soil moisture. Softwood cuttings of juvenile stems rooted at high frequency (up to 93% success). Rooting was best among cuttings from terminal positions on stock plants treated with a solution containing 1-naphthalenacetic acid (NAA). Genetic analyses of 24 populations of *N. aquatica* revealed moderate differentiation among populations and minimal regional structure. I conclude that *N. aquatica* has potential for use in urban landscapes prone to wide fluctuations in soil moisture, provided that episodes of water deficit are not severe or extended. Propagation of clonal selections of juvenile trees is possible by using stem cuttings. Minimal regional genetic structure suggests that clonal selections from any part of the range could be marketed without concerns about influencing the genetic structure of any particular region.

CHAPTER 1. GENERAL INTRODUCTION

Introduction

Wide fluctuations in soil moisture caused by mismanagement of water and climatic events can be a major stressor for trees in managed landscapes. In urban environments, the rapidity of the onset and the intensity of the stress are frequently exacerbated by high temperatures, soil compaction, and limited rooting volumes (Flückiger and Braun, 1999; Jim, 1998; Pouyat et al., 1995). Trees native to periodically flooded wetlands tolerate periods of flooding punctuated by soil drying and may be valuable in managed landscapes where soil moisture fluctuates widely. Although restricted to saturated or seasonally inundated soils in the wild, model wetland trees like *Taxodium distichum* L. (bald cypress) and *Alnus maritima* Marsh. (seaside alder) show a surprising degree of tolerance to dry soils and perform well in urban landscapes (Nash and Graves, 1993; Schrader et al., 2005).

Nyssa aquatica L. (water tupelo) is a large, deciduous tree native to continuously and seasonally flooded swamps and floodplains throughout the lower Mississippi River Valley, Gulf Coastal Plain, and Atlantic Coastal Plain, where it is an economically and ecologically important forest species. Seedlings of *N. aquatica* grow quickly (up to 2.4 m in the first year; personal observation), and mature trees can reach 30 m (Johnson, 1990). Leaves are large (10 to 20 cm long) with entire or toothed margins and yellow fall color. The polygamodioecious trees produce inconspicuous greenish-white clusters of flowers in the spring and large (2.4-cm-long) purple drupes in the fall (Dirr, 1998). When trees have been inundated, trunks have a distinctive flared buttress. Although not recognized for distinct ornamental characteristics, *N. aquatica* has potential for landscape use based on its tolerance to soil moisture extremes in the wild. *Nyssa aquatica* thrives in soils in which seasonal

flooding is punctuated by soil drying. In coastal wetlands, for example, the water table can fluctuate more than 2 m within a few weeks (Christensen, 1988).

The exceptional flood tolerance of *N. aquatica* is well defined (Gravatt and Kirby, 1998; Harms, 1973; Hook and Stubbs, 1967; Keeley, 1979). However, its tolerance to water deficit has been questioned based on inducible morphological traits that improve flood tolerance (*e.g.* aerenchyma, large intercellular spaces within cambial regions) but may increase susceptibility to desiccation in dry soils (Crawford, 2008; Keeley, 1979). For some flood-tolerant trees, preexposure to flooding increases the risk of mortality upon subsequent exposure to dry soil (Miao et al., 2009), although this is not the case for all wetland species (Elcan and Pezeshki, 2002). If seedlings of *N. aquatica* have increased sensitivity to water deficit after flood events, their use in managed landscapes with widely fluctuating soil moisture would be limited. An understanding of the responses of *N. aquatica* to multiple extreme hydrologic events within a season is essential prior to recommendation for landscape use. In addition, information about differences in tolerance to soil moisture extremes among provenances of *N. aquatica* would be useful for making selections for horticulture. Protocols for asexual propagation are also needed for multiplication of superior selections.

Although *N. aquatica* is widespread in the southeastern United States, native populations are declining as a result of human encroachment and habitat destruction (Stallins et al., 2009). In the Apalachacola River Basin, populations of *N. aquatica* have declined by 20% in the past 30 years (Darst and Light, 2008). As wetland ecosystems degrade, the use of *N. aquatica* in urban horticulture may provide additional habitat for the species. Baseline information about the population genetic structure of the species across its range could aid in decisions about where to market specific selections. In addition, the species could be viewed

as a model species for other large, widespread wetland trees for which regional-scale population genetic data is not currently available.

My research examined some critical questions about the potential for use of *N. aquatica* in urban horticulture. The goals of this research were

1. to characterize the effects of water deficit and extreme fluctuations in soil moisture on height growth, biomass, gas exchange, and water relations of seedlings of *N. aquatica*;
2. to determine whether seed source affects tolerance of seedlings of *N. aquatica* to water deficit or fluctuations in soil moisture;
3. to determine whether seedlings of *N. aquatica* can be propagated asexually via stem cuttings and how auxin type, auxin concentration, and cutting position influence rooting; and
4. to assess the regional genetic structure of wild populations of *N. aquatica*.

Although many factors will inevitably influence the potential for *N. aquatica* in ornamental horticulture, including cold hardiness, disease resistance, and ornamental appeal of specific selections, the questions in this research provide a basis upon which to support or reject further evaluation of the species.

Dissertation Organization

This dissertation contains four manuscripts. The manuscripts in Chapter 2 and 3 are formatted for submission to the *Journal of the American Society for Horticultural Science* and *Castanea*, respectively. The manuscript in Chapter 4 was published in the *Journal of Environmental Horticulture* and is formatted for that journal. Chapter 5 is formatted for submission to *Aquatic Botany*. A brief literature review and general conclusions are also

included. Appendices contain supplemental information pertaining to the study in Chapter 5 and preliminary data about the graft compatibility of *N. aquatica* and *Nyssa sylvatica*.

Literature Review

Responses of wetland plants to water stress

The capacity of wetland plants to tolerate low-oxygen conditions induced by flooding distinguishes them from many plants in other ecosystems. However, in a number of wetlands, flooding is ephemeral, and plants that thrive in these ecosystems must also have adaptations to tolerate water deficit (Braendle and Crawford, 1999). Although tolerance to water deficit can be important in these fluctuating environments, a more important adaptation of many wetland species is the capacity to avoid post-anoxic injury, which occurs when oxygen is reintroduced after a period of anoxia. Following anoxia, reactive oxygen species are formed. Reintroduction of oxygen can also trigger the formation of toxic acetaldehyde from hydrogen peroxide and ethylene accumulated as an end-product of anaerobic respiration (Crawford and Braendle, 1996; Monk et al., 1987). Many wetland plants avoid post-anoxic injury by oxygenating submerged tissues and preventing anoxia at the outset of flooding (Braendle and Crawford, 1999).

Morphological features, such as aerenchyma, hypertrophied lenticels, and adventitious roots that proliferate at the surface of the water where oxygen is less limiting, allow wetland plants to avoid anoxia. Large intercellular spaces within cambial initials and ray cells of differentiating phloem have also been implicated in flood tolerance of wetland trees (Hook and Brown, 1972). Aerenchyma and intercellular spaces provide low-resistance channels for air movement to submerged tissues, facilitating intake of oxygen from stomata and lenticels and efflux of injurious gasses (Hook and Scholtens, 1978). Some flood-tolerant

plants have higher rates of stomatal conductance during and following flooding (Elcan and Pezeshki, 2002), which may represent an adaptive strategy for maximizing oxygen intake. For plants native to periodically flooded wetlands, morphological adaptations to flooding are often induced upon flooding (Keeley, 1979; Loreti and Oesterheld, 1996). During the initial stages of flooding when aeration systems may not be fully developed, efficient anaerobic respiration can also be an important characteristic for wetland plants (Keeley, 1979).

Many wetland plants are considered sensitive to water deficit based on growth or competitiveness with mesic or xeric plants. In managed landscapes, however, these factors are rarely as important as a plant's capacity to survive and maintain ornamental appeal during and following stress events. Perennial wetland species frequently have multiple mechanisms that allow them to survive periods of water deficit. One common mechanism is by decreasing transpirational demand or increasing uptake of water to avoid desiccation. Desiccation-avoidance mechanisms for many wetland species include leaf abscission, increased root:shoot ratio, and decreased transpiration rates. Desiccation-tolerance strategies observed in wetland plants involve mechanisms that permit survival at suboptimal water potentials, including increases in cellular elasticity and osmotic regulation (Nash and Graves, 1993; Romanello et al., 2008; Touchette et al., 2007). Although these mechanisms can improve survival during water deficit, plants with certain drought-tolerance mechanisms (*e.g.* excessive leaf drop) may be undesirable or unacceptable in a managed landscape because of reduced in visual appeal during periods of water deficit (Zollinger et al., 2006).

Tolerance to the stresses of water deficit and flooding is influenced by intensity, timing, duration, and rapidity of the onset of the stress, among other factors (Kramer and Boyer, 1995). Preexposure to stress can also influence response to that stress in the future.

This phenomenon is well characterized for plants exposed to water stress. Increased tolerance after sublethal exposure to water deficit has been associated with osmotic regulation, greater stomatal sensitivity, increases in cellular elasticity, changes in membrane properties, and modification of dry weight partitioning (Ackerson, 1980; Ansley et al., 1992; Kozlowski and Pallardy, 2002; Unterscheutz et al., 1974). For flood-tolerant plants, concerns have also been raised about the effects of flood-preconditioning on tolerance to water deficit, based on the fact that roots containing aerenchyma have reduced capacity to absorb water and nutrients in drained soils (Koncalova, 1990). In addition, root-aeration systems provide a low-resistance pathway for water movement out of the roots (Keeley, 1979). An increase in stomatal conductance following flooding, which has been seen for both *T. distichum* (bald cypress) and *Carya illinoiensis* (Wangenh.) K. Koch (pecan) (Elcan and Pezeshki, 2002; Smith and Huslig, 1990), may also heighten the risk of desiccation in drying soils. Few studies have examined the effects of flood-preconditioning on drought tolerance, and these studies show contrasting results (Elcan and Pezeshki, 2002; Miao et al., 2009; Smith and Huslig, 1990).

Tolerance of *Nyssa aquatica* to flooding

Nyssa aquatica is extremely flood tolerant and can grow in deep, stagnant water, but growth is best if water is oxygenated (Harms, 1973). Some variation in flood tolerance has been observed for seedlings of *N. aquatica*. Seedlings originating from continuously flooded bottomlands have been shown to have a greater capacity for anaerobic respiration and greater tolerance to intermittent flooding than seedlings from alluvial sources (Hook and Stubbs, 1967; Keeley, 1979). Many of the flood-tolerance mechanisms of *N. aquatica* depend on the duration of flooding and include upregulation of anaerobic respiration in the short term and

morphological adaptations to increase oxygenation of submerged portions in the long term. At the onset of flooding, anaerobic respiration causes increases in ethylene production, accompanied by dieback of the original root system (Keeley, 1979). Slight reductions in photosynthesis and stomatal conductance, as well as increased starch concentrations in roots, stems, and leaves, have also been observed for seedlings at the onset of flooding (Gravatt and Kirby, 1998). New secondary roots, which have little suberization and no Casparian strip but contain abundant intercellular spaces within the cortex, are formed within days after plants are inundated (Hook and Scholtens, 1978). These roots can maintain high rates of anaerobic respiration (Keeley, 1979). Morphological adaptations are induced if flooding continues. These include proliferation of hypertrophied lenticels above the water line and development of a root system that contains aerenchyma, respires aerobically, and oxygenates the rhizosphere (Hook and Scholtens, 1978; Keeley, 1979).

Tolerance of *Nyssa aquatica* to water deficit

Seedlings of *N. aquatica* are considered to be sensitive to water deficit based on growth reductions, propensity to wilt in suboptimal soil moisture, and slow recovery of stem water potential after rewatering (Dickson and Broyer, 1972; Dickson et al., 1965). However, seedlings have at least two mechanisms that confer some degree of tolerance to water deficit: reduction of transpirational water loss as soil dries, likely through stomatal closure, and increased root:shoot ratio (Dickson and Broyer, 1972).

Propagation of *Nyssa aquatica*

Nyssa aquatica is propagated commercially by seed (Johnson, 1990), but information about asexual propagation is lacking. As is the case for ornamentally valuable *Nyssa* species, phenotypic variation for seedlings within and among seed sources can be large (Dummer,

1968; Hook and Stubbs, 1967). For *N. aquatica* to be marketed successfully as an ornamental species for horticulture, specific genotypes would need to be identified and multiplied asexually. Propagation from stem cuttings is widely practiced in ornamental horticulture because of its technical simplicity (Hartmann et al., 2002) and has been successfully used for species closely related to *N. aquatica* (Dummer, 1968). For *N. sylvatica*, up to 100% of juvenile stem cuttings rooted when treated with 8000-ppm indole-3-butyric acid (IBA), an auxin that aids in initiation and growth of adventitious roots and is widely available in the nursery industry (Dummer, 1968; Hartmann et al., 2002). Application of synthetic auxin may also promote rooting in *N. aquatica*, although many other factors play roles, including the age of the cutting and the position on the stock plant from which it was taken (Morgan et al., 1980; O'Rourke, 1944), and environmental factors (Eliasson, 1980; Kristiansen et al., 2005).

Historical biogeography

Fossil evidence suggests several *Nyssa* species were widespread in North America in the Tertiary period, and comparisons of fruit morphology suggest *N. aquatica* may be the remaining species stemming from this previously widespread subgroup (Eyde, 1997). Today, the range of *N. aquatica* is restricted to freshwater wetlands of the Lower Mississippi River Valley and Gulf and Atlantic Coastal Plains as far north as southern Virginia. Pleistocene glaciation events have been implicated in the southward advance of the distribution of *N. aquatica* as well as in many other southeastern flora and fauna (Avise, 1996; Eyde, 1963, 1997). These glacial advances and retreats influenced not only distributional patterns but also genetic structuring within the species. Phylogeographic analyses based on data from allozymes, mitochondrial or chloroplast DNA, and nuclear DNA

of many fauna (Avise, 2000) and some flora (Lickey and Walker, 2002; Mylecraine et al., 2004) show a highly predictable pattern of regional population differentiation between the Gulf Coast and the Atlantic Coast, with a split occurring near the Apalachicola River. One widely accepted explanation for this separation between Atlantic and Gulf populations is that saline estuaries and salt marshes existed around the Floridian peninsula during interglacial periods, creating a physical barrier to gene flow for temperate, freshwater species (Avise, 1996). Another hypothesis is that recent migration patterns have created the genetic differentiation between the two coastal plains in the absence of a physical barrier to gene flow (Baer, 1998). This theory, however, is based on two stepping stone models that produce population differentiation based on differing migration routes from the Floridian peninsula. Because the range of *N. aquatica* does not include peninsular Florida, a recent migration hypothesis is unlikely for this species. Drainage isolation and connection caused by glacial advances and retreats also likely affected freshwater species in this area (Avise, 1996), but the genetic consequences of these events are less widespread than those resulting from a possible physical separation of freshwater ecosystems in the Atlantic and Gulf Coastal Plains.

Life history traits

Nyssa aquatica is polygamodioecious (*i.e.* individual trees have staminate and perfect or pistillate and perfect flowers), and although its mating system is largely uncharacterized, the sexual structure of the plant indicates the species is primarily outcrossing.

We are unaware of any published research specifically addressing pollination of *N. aquatica*. However, several researchers have speculated that pollen is distributed primarily

by wind (Eyde, 1997; Shea et al., 1993). Wind-outcrossed species typically show less genetic differentiation and higher migration rates than animal-outcrossed or self-pollinated species (Hamrick and Nason, 1996). Thus, if pollen-migration rates among populations are high, populations of *N. aquatica* may show little population or regional differentiation even if other influences would tend to increase differentiation.

Mature drupes are produced when trees are around 30 years old (Johnson, 1990 and are primarily disseminated by water (Eyde, 1997). Seed germination requires oxygen (Shunk, 1939), so new seedlings sprout only when water levels recede. Thus, seasonal changes in hydrology are crucial for the establishment of new populations of *N. aquatica*.

Benefits and limitations of using ISSR markers

Inter-simple-sequence-repeats (ISSRs) are PCR-generated sequences obtained using long, anchored di- or tri-nucleotide repeats. These markers produce highly polymorphic banding patterns that are often variable enough to be used effectively within species (Robinson et al., 1997; Schrader and Graves, 2004a; Wolfe and Liston, 1998). In addition, ISSRs also have the advantage of sampling a large portion of the genome and therefore may be less biased than markers based on a single sequence or on one or a few genes (Schrader and Graves, 2004b). Primers can be chosen without prior knowledge of the genome and therefore require little development time or expense. Other advantages of ISSRs include the need for only small amounts of DNA and small reaction volumes, technical simplicity, and cost effectiveness (Wolfe et al., 1998; Yang et al., 1996). Because ISSRs are a dominant marker, however, they cannot be used to infer allele frequencies directly, which results in less power to infer genetic relationships. Assumptions have to be made about the origin of alleles, and band scoring is sometimes ambiguous. However, results of genetic studies with

dominant markers are generally in accordance to those performed with codominant markers, especially when a large number of loci are used in the analyses (Hollingsworth and Ennos, 2004; Simmons et al., 2007; but see Levsen et al., 2008).

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CHAPTER 2. ROOT-ZONE MOISTURE BEFORE WATER DEFICIT AFFECTS STRESS RESPONSE IN TWO *NYSSA* SPECIES

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Abstract. Many wetland trees thrive in habitats that experience cycles of flooding and soil drying and could be well adapted to urban landscapes in which soil moisture fluctuates widely. We conducted two experiments to determine the effects of wide fluctuations in soil moisture on growth, biomass, gas exchange, and leaf water potential of the wetland trees *Nyssa aquatica* L. (water tupelo) and *Nyssa ogeche* Bartram (Ogeechee tupelo). For each experiment, plants received two consecutive six- to eight-week soil moisture treatments, ranging from flooding to water deficit. Both species were tolerant of fluctuations in soil moisture, but were sensitive to water deficit early in the growing season. Root-zone moisture before extreme hydrological events did not affect growth or biomass responses during extreme events. However, water deficit applied late in the season caused greater reductions in visual quality (*e.g.* tip dieback, leaf scorch) and gas exchange when plants were pretreated with moderate soil moisture than when pretreated with flooding. The photosynthetic rate of plants exposed to moderate soil moisture before water deficit was only 30% that of the control at the end of the season, whereas plants pretreated with flooding or exposed to continual water deficit showed no reductions in photosynthesis. *Nyssa aquatica* and *N. ogeche* have potential for use in urban landscapes, but their use may be limited to soils in which water available early in the growing season. Pretreatment with flooding may be

beneficial before transplanting seedlings or in landscapes where water deficit is likely late in the season.

Introduction

Increasing frequency and intensity of flood and drought events associated with global climate change is a concern in both natural and managed landscapes (Brazel et al., 2000; Huntington, 2006; McCabe et al., 2004). Wide oscillations in soil moisture within a growing season have been linked to high mortality rates for mesic forest trees (Auclair, 1993) and can be especially problematic in urban landscapes where water mismanagement and limited rooting volumes are common. Trees indigenous to periodically flooded wetlands regularly withstand cycles of flooding and soil drying (Keeland et al., 1997; Mitsch et al., 1979) and may be better suited than trees from mesic origins to landscapes with wide fluctuations in soil moisture. However, despite a vast literature detailing responses of wetland plants to isolated flood or drought events (Luo et al., 2008; Parolin, 2001; Touchette et al., 2007; Vasellati et al., 2001), little is known about the effects of fluctuations in soil moisture within a single growing season. Understanding the effects of hydrologic fluctuations is important for landscape plants that will likely be exposed to fluctuations in soil moisture that may not coincide with natural hydrological cycles.

Adaptations to flooding and drought generally involve different responses, but questions have been raised about whether these adaptations are mutually exclusive (Crawford, 2008). Some authors suggest that morphological and physiological adaptations to flooding could disadvantage plants in drained soils (Braendle and Crawford, 1999; Crawford, 2008; Keeley, 1979). Roots that produce aerenchyma to facilitate oxygenation in flooding

have reduced capacity for water and nutrient uptake and increased risk of desiccation in mesic soils (Keeley, 1979; Koncalova, 1990). Some flood-tolerant plants also have elevated stomatal conductance (g_s) in flooded soils that may not immediately decline upon soil drying, further increasing the risk for desiccation (Anderson and Pezeshki, 2001; Li et al., 2004). Other adaptations that could influence the response of flood-adapted species to water deficit include shallow rooting and rapid stem elongation that would potentially increase transpirational demand and limit the volume from which plants are initially able to extract water (Crawford, 2008). Recent work supports the conclusion that, at least for some wetland trees, a period of flooding followed immediately by water deficit is more detrimental to survival and growth than water deficit followed by flooding (Miao et al., 2009). However, it is possible that adaptations to water deficit, such as leaf abscission and stomatal closure could also influence survival upon sudden flooding by reducing carbohydrate reserves available to the plant when anaerobic respiration is occurring and the efficiency of energy production is reduced. Whether root-zone moisture before extreme events affects response to water deficit or flooding will likely depend on the specific suite of tolerance mechanisms as well as whether traits are plastic or constitutive.

Nyssa aquatica L. (water tupelo) and *Nyssa ogeche* Bartram (Ogeechee tupelo) are wetland trees that have potential for increased use in managed landscapes. In the wild, *N. ogeche* is restricted to wet soils (Kossuth and Scheer, 1990), but *N. aquatica* is found in periodically and continuously flooded soils (Johnson, 1990). *Nyssa aquatica* can withstand long periods of continuous flooding, but can also survive short periods of water deficit (Dickson et al., 1965; Dickson and Broyer, 1972). Flood adaptations include temporary induction of anaerobic respiration (Keeley, 1979), large intercellular spaces in the cambial

tissue and phloem derivatives (Hook and Brown, 1973), and production of aerenchymatous roots and hypertrophied lenticels (Hook et al., 1970; Hook et al., 1971; Keeley, 1979). Genetic variation in flood tolerance has also been noted for seedlings from different hydrologic environments (Hook and Stubbs, 1967). In response to water deficit, seedlings of *N. aquatica* slow growth and decrease transpiration, but little else is known about their response to limited moisture (Dickson et al., 1965; Dickson and Broyer, 1972). We are unaware of published studies that describe directly the tolerance of *N. ogeche* to flooding or water deficit.

We performed two experiments to determine the effects of wide fluctuations in soil moisture on biomass, leaf gas exchange, and leaf water potential of seedlings of *N. aquatica* and *N. ogeche*. Because of its distribution in the wild, we hypothesized that *N. aquatica* would have greater tolerance to fluctuations in soil moisture than *N. ogeche* and that root-zone moisture before flooding or water deficit would influence biomass and physiological responses to extreme hydrological events for both species. Because differences have been noted in flood tolerance of seedlings from different seed sources, we also sought to determine whether different provenances of *N. aquatica* would react differently to fluctuations in soil moisture.

Materials and Methods

Experiment 1

Experiment 1 was designed to gather baseline information about growth, stomatal responses, and water relations of seedlings of both species in response to various hydrologic regimes and extreme fluctuations in soil moisture.

Plant material. Seedlings of *N. aquatica* were grown from seeds collected in 2005 from trees growing in a seasonally inundated floodplain in Union County, IL, and along the banks of the Suwannee River in Levy County, FL. Seedlings of *N. ogeche* were grown from seeds collected in 2005 from Liberty County, FL. All seeds were cold-stratified in a minimally heated greenhouse and germinated in the spring of 2006. Seedlings were transplanted into 1.8-L pots (15.24-cm top diameter) in soilless medium (Sunshine LC1 Mix, Sun Gro Horticulture, Seba Beach, Alberta, Canada) when one true leaf had formed, and plants were irrigated with tap water once weekly for four weeks, then fertilized once weekly with Miracle Gro liquid acid fertilizer at 1187 $mg \cdot kg^{-1}$ N (30N-4.4P-8.3K; MirAcid, Scotts, Marysville, OH) for two weeks before treatments were applied.

Treatments. Treatments were imposed in two consecutive phases. Phase 1 started on 30 June 2006 and lasted 53 d, and Phase 2 started 23 Aug. 2006 and lasted 59 d. Seedlings were randomly assigned to one of the following Phase 1-Phase 2 treatments: partial flood-partial flood, flood-flood, water deficit-water deficit, partial flood-water deficit, flood-water deficit, or water deficit-flood. Seedlings that were partially flooded during both phases were the control. Partially flooded seedlings were placed in containers in which tap water was maintained at 2.5 cm above the bottom of the pot, so the lower portion of the medium remained saturated. For the flood treatment, pots were placed inside 2.8-L containers, and water level was maintained at 3 cm above the surface of the media. Water deficit was applied in a cyclic manner by allowing soil to dry followed by irrigation to container capacity. The first time water deficit was applied, stomatal conductance (g_s) of seedlings was measured daily, and seedlings were irrigated when g_s fell below 0.100 $mol \cdot m^{-2} \cdot s^{-1}$, approximately half of the pre-stress value. This corresponded with midday wilting and soil

moisture of approximately 0.20 $m^3 \cdot m^{-3}$ in the top 6 cm of the pot. After the first cycle of water deficit, medium moisture was monitored daily, and plants were irrigated when moisture content fell below 0.20 $m^3 \cdot m^{-3}$. To prevent nutrient deficiencies, 250 mL liquid fertilizer was added to each pot when irrigated at same formulation and rate as the initial fertilization, and seedlings in the partially flooded and flooded treatments were fertilized once monthly.

Environmental conditions. The experiment was conducted in a glass-glazed greenhouse in Ames, IA, with no supplemental irradiation. Photosynthetically active radiation (*PAR*) at canopy level was measured daily at midday using a Li-1600 quantom sensor (LiCor, Lincoln, NE). Average midday *PAR* was 574 $\pm$ 45 $\mu mol \cdot m^{-2} \cdot s^{-1}$ during Phase 1 and 589 $\pm$ 81 $\mu mol \cdot m^{-2} \cdot s^{-1}$ during Phase 2. Greenhouse temperature was logged hourly during the study period using a Thermochron i-Button (Dallas Semiconductor, Dallas, TX). Average daily minimum/maximum temperatures were 21/26° C during Phase 1 and 21/23° C during Phase 2.

Data collection. Stomatal conductance of the newest fully expanded sunlit leaf of each seedling was measured once per week between 1000 and 1400 HR with a steady-state porometer (Li-1600, LiCor, Lincoln, NE). During Phase 1, daily measurements of g_s were made for seedlings in the water deficit treatment as a check of the physiological stress of each plant as the medium dried to the prescribed moisture. Predawn leaf water potential ($\Psi_{predawn}$) was determined for four seedlings from each species or provenance x treatment group immediately before initiating Phase 2 treatments (22 Aug. 2006) and from all seedlings on the day of harvest using a Scholander-type pressure chamber (PMS Instrument, Corvallis, Oregon). For predawn measurements, leaves were sampled between 0500 and 0600 HR.

Height of *N. aquatica* seedlings (root collar to terminal bud) was measured before treatments began and once weekly during the treatment period, and relative height growth rates were calculated for individual trees each week using:

$$RGR_{height} = [\ ln(H2) - ln(H1)] / \Delta d;$$

where H2 = ending height, H1 = beginning height, and Δd = the time interval (d) between the beginning and ending measurements.

Seedlings were harvested on 21 Oct. 2006, and leaves, shoots, and roots were separated. Leaf area was determined by passing fresh leaves through a Li- 3100 leaf area meter (Li-Cor, Lincoln, NE). For each seedling, total number of leaves was also recorded, and average leaf size was determined by dividing total leaf area by the number of leaves. Medium was washed from roots, and roots, shoots, and leaves were dried at 70° C for 48 hours before weighing.

Experimental design and statistical analyses. The experiment was a completely randomized design arranged as a factorial with five replicates of each species or provenance x treatment combination. All data were analyzed using a general linear model analysis of variance (ANOVA) in SAS (v.9, SAS Institute, Cary, NC). Because of inherent differences in growth and form, biomass and leaf area data were analyzed separately for each species. For *N. ogeche*, data were analyzed as a one-way ANOVA with six Phase 1 - Phase 2 treatments. Data for *N. aquatica* were analyzed as a two-way ANOVA with two provenances and six treatments. Mean separations were conducted using the Ryan-Einot-Gabriel-Welsch multiple range test, and contrasts were used to test specific comparisons of interest. Because differences were not apparent between species for physiological parameters, data for both species were analyzed together as a two-way ANOVA with three

taxonomic groups (*N. ogeche*, *N. aquatica* from Illinois, *N. aquatica* from Florida) and six treatments. For data taken over time, separate analyses were performed for each day measurements were taken.

Experiment 2

Experiment 2 was designed to examine the effects of preexposure to a range of soil moisture on biomass, gas exchange, and water relations of *N. aquatica* from different provenances in flooding, water deficit, or partial flooding.

Plant material. Plants of *N. aquatica* were grown from stem cuttings taken in July 2007. Cuttings were taken from seedlings grown from seeds collected from the same locations as in Expt. 1 and from a periodically flooded stand in Aiken Co., SC. In Sept. 2007, rooted cuttings were transplanted into 2.8-L pots in soilless medium composed of 60% processed pine bark, 30% sphagnum peat, and 10% perlite (Conrad Fafard mix #51, Agawam, MA). Plants were overwintered in a glass-glazed greenhouse in Ames, IA, that was kept above 10 C (daily average = 15 C) and provided with no supplemental irradiation. Starting in May 2008, trees were irrigated daily with tap water, and fertilized once each week with 385 mg $N \cdot L^{-1}$ Peters Excel All-Purpose 21N-2.2P-16.6K (Scotts, Marietta, GA) until treatments were initiated. Plants were transplanted into 7.6-L pots (21 cm diameter, 22 cm tall) in June 2008 and allowed to establish for two weeks before treatments began.

Treatments. Treatments were applied in two consecutive phases. Phase 1 began on 2 July 2008 and lasted 39 d, and Phase 2 began on 11 Aug. 2008 and lasted 45 d. Phase 1-Phase 2 treatments were applied as a complete factorial, with each plant receiving partial flood, flood, or water deficit treatment during each of the phases. For the partial-flood treatment, pots were set in plastic saucers (20-cm bottom diameter, 9-cm tall) filled with

water so the lower portion of the medium was kept saturated. Water in the saucers was replenished daily as needed. Plants in the flooded treatment were placed in 11-L buckets filled with tap water. Flood water was maintained at 5 cm above the medium surface. Before treatments were imposed, 2 cm of coarse sand was spread on the surface of the medium in each pot to stabilize inundated medium. During Phase 1, water deficit was applied by withholding water from individual pots until medium moisture in the upper 6 cm fell below 0.10 $m^3 \cdot m^{-3}$ and then adding 100 ml, which allowed us to maintain medium moisture below 0.20 $m^3 \cdot m^{-3}$. During phase 2, plants were larger, so the threshold for adding water was increased to 0.20 $m^3 \cdot m^{-3}$. Plants in the flooded and partially flooded treatments were removed from their containers and fertilized weekly with 385 mg $N \cdot L^{-1}$ Peters Excel All-Purpose 21N-2.2P-16.6K. Excess fertilizer was allowed to drain before plants were returned to their containers. To maintain adequate nutrient availability and avoid buildup of excess salts, plants in the water deficit treatment received half-strength liquid fertilizer every other time water was added.

Environmental conditions. Measurements of temperature, relative humidity, and *PAR* were logged hourly during the study period with a CR10 datalogger (Campbell Scientific, Logan, UT) equipped with two quantum sensors placed at canopy level (Li-190, LiCor, Lincoln, NE) and a temperature-relative humidity sensor (CS500-L, Campbell Scientific, Logan, UT). Mean daily minimum/maximum temperatures were 23/32° C and 22/28° C for Phase 1 and Phase 2, respectively. Photosynthetically active radiation at midday averaged 692 $\mu mol \cdot m^{-2} \cdot s^{-1}$ during Phase 1 and 460 $\mu mol \cdot m^{-2} \cdot s^{-1}$ during Phase 2.

Data collection. Soil moisture in the upper 6 cm of medium was measured daily using a Theta probe (Decagon Devices, Pullman, WA). Predawn and midday water potential

of new, fully expanded leaves were monitored using a Scholander-type pressure chamber (PMS Equipment, Albany, OR). Measurements of midday water potential were taken for one plant from each provenance x Phase 1 x Phase 2 group between 1200 and 1400 HR on days 3, 13, 29, 38, 65, 71, 76, and 84. Measurements of predawn water potential were taken between 0500 and 0600 HR 1 d before treatments were initiated and on the last day of Phase 1 and Phase 2 for one plant from each provenance x Phase 1 x Phase 2 group.

Photosynthesis (P_n) and g_s were measured on the newest fully expanded leaf of each plant one day before treatments were imposed and weekly throughout the experiment using a Li-6400 Photosynthesis System (LiCor, Lincoln, NE). For all measurements, chamber conditions were as follows: *PAR* = 600 µmol·m^{-2}·s^{-1}, chamber CO_2 = 400 µmol·mol^{-1}, and relative humidity = 50%.

Plants were harvested on 25 Sept. 2008. Leaf area and dry weights were obtained as outlined for Expt. 1. In addition, one 1-cm-long section from a root measuring 1 to 3 mm in diameter was taken from 10 cm behind the root tip for each plant for examination of aerenchyma. Root sections were fixed in formalin-acetic acid-alcohol (FAA), dehydrated in an alcohol series, and embedded in paraffin (Berlyn and Miksche, 1976). Roots were cut transversally into 15-µm thick sections, stained with Safranin O and Fast Green (Jensen, 1962), and examined under a stereo microscope (Nikon SMZ 800; Nikon, Tokyo, Japan) equipped with a digital camera (Nikon CoolPix 995; Nikon, Tokyo, Japan). Percentage aerenchyma within the cortex was estimated from a digitized image of each section by comparing the area occupied by air space and the total area of the cortex.

Statistical design and analysis. The experiment was a split plot design with provenance as the main factor and Phase 1 and Phase 2 treatments as subplot factors. Plants

were organized into three blocks with one replication each to account for variation in greenhouse conditions. Analyses of all variables were performed using a MIXED model analysis of variance in SAS (v.9, SAS Institute, Cary, NC). Main effects of provenance, Phase 1 treatment, and Phase 2 treatment as well as all interactions were tested. Block was treated as a random effect. Mean separations were performed using Tukey's honestly significant difference test. Variables were transformed as needed to satisfy assumptions of homogeneity and approximate normality. For data taken over time, separate analyses were performed for each day measurements were taken. Stomatal conductance of individual plants subjected to water deficit in Phase 2 was regressed against measurements of medium moisture for the first 16 d of Phase 2, during which time soil moisture in all pots dried to the prescribed moisture. Mean regression lines were calculated by averaging coefficients among individuals within groups. Differences in regression coefficients among treatments and provenances were tested using a MIXED model as described above.

Results

Experiment 1

Predawn water potential. At the end of Phase 1, $\Psi_{predawn}$ was -0.38 MPa for seedlings subjected to water deficit and -0.21 MPa for seedlings that were flooded or partially flooded ($P = 0.0014$). However, at the end of Phase 2, $\Psi_{predawn}$ of seedlings treated with continuous flooding, continuous water deficit, or a combination of flooding and water deficit was similar to that of seedlings that were partially flooded throughout the experiment. Only $\Psi_{predawn}$ of seedlings subjected to partial flooding in Phase 1 and water deficit in Phase 2 was less than that of the control (Table 1).

Stomatal conductance. Throughout the experiment, g_s was similar for both species and provenances of *N. aquatica*, so values presented are means of the three taxonomic groups. Conductance of seedlings in the water deficit treatment fell to half that of seedlings in the flooded and partially flooded treatments within three weeks of when treatments were initiated (Figure 1). No differences were detected between the flooded and partially flooded treatments until the sixth week of the study, at which time g_s of seedlings in the flooded treatment exceeded that of seedlings in the partial flood and water deficit treatments by 22% and 66%, respectively (Figure 1).

Seedlings subjected to water deficit in Phase 2 had similar g_s one week after Phase 2 began, regardless of differences in soil-moisture conditions during Phase 1. In contrast, g_s of seedlings subjected to water deficit before flooding did not recover to the rate of continuously flooded seedlings for three weeks. By the third week of Phase 2, seedlings that were partially flooded or flooded during Phase 2 had greater g_s than seedlings subjected to water deficit in Phase 2, with the exception of seedlings that were flooded before being exposed to water deficit (Figure 2). This trend continued through the end of the study, although declining rates of g_s across all treatments limited our power to detect differences during the final three weeks.

Height growth. Seedlings of *N. aquatica* from Florida were taller than those from Illinois (104 cm and 98 cm, respectively, at the end of the season; $P < 0.0001$), but seedlings within the same treatment had similar RGR_{height} throughout the experiment. Relative height growth rate was greatest at the beginning of the study and declined over the course of the experiment (Figure 3). By the third week, seedlings in the water deficit treatment had 31% smaller RGR_{height} than those in the control. By the fourth week, RGR_{height} of seedlings in the

flooded treatment was also depressed compared with the control but remained greater than seedlings in the water deficit treatment throughout Phase 1. During Phase 2, RGR_{height} was only 38% of its value during Phase 1, and no consistent differences were detected among treatments.

Biomass and leaf area. Seedlings of *N. aquatica* from Florida produced 40% more total biomass and had 36% larger leaves than those from Illinois (Table 2). However, treatments affected biomass and leaf area similarly, so values are given as means of the two provenances except where otherwise stated.

Water deficit applied at any time during the experiment reduced total dry biomass of *N. aquatica* by 49%, whereas continuous flooding reduced biomass by 27%, compared with the control (Table 2). Seedlings that were not exposed to water deficit or flooding had 69% more leaf area than those that were subjected to water deficit and/or flooding (Table 2). Pretreating seedlings with water deficit or flooding before water deficit decreased both stem weight and leaf size by 36% compared with seedlings pretreated with partial flooding (Table 2). For seedlings subjected to both water deficit and flooding, the sequence of events influenced leaf mass, but not any other measured trait. At the end of the experiment, seedlings exposed to water deficit before flooding had 15% greater leaf mass than seedlings exposed to flooding before water deficit (Table 2).

Root growth in continuously flooded conditions differed between provenances of *N. aquatica*. Seedlings from Florida produced 84% more root mass when subjected to continuous flooding than when subjected to flooding during only one phase of the experiment (Table 3). However, for seedlings of *N. aquatica* from Illinois, root mass was similar for seedlings that were subjected to flooding for any length of time (Table 3).

Continuous water deficit reduced total biomass of *N. ogeche* seedlings by 72% compared with the control (Table 2). Seedlings in the control produced 83% more leaf area than seedlings exposed to continuous water deficit, but the high mortality rate for seedlings in the water deficit treatment limited the power to detect differences between these two groups. Neither pretreatment nor sequence of water deficit and flood events influenced biomass, leaf area, or leaf size of *N. ogeche* seedlings. Seedlings of *N. ogeche* had a small root:shoot ratio (0.25) in all treatments. Root:shoot ratio of seedlings of *N. aquatica* was 40% larger than that of *N. ogeche* ($P < 0.0001$).

Mortality. Some seedlings of both *N. aquatica* and *N. ogeche* died when exposed to water deficit during Phase 1. Twenty percent of *N. aquatica* from Illinois and 40% of *N. ogeche* in the water deficit treatment died within five weeks of treatment initiation.

Experiment 2

Water potential. Plants subjected to water deficit had lower Ψ_{midday} than plants in the partially flooded or flooded treatment throughout Phase 1. Plants that were partially flooded also had consistently lower Ψ_{midday} than plants that were flooded and frequently wilted in the middle of the day. At the end of Phase 1, Ψ_{midday} was -1.15, -0.84, and -0.47 MPa for plants in the water deficit, partial-flood, and flood treatments, respectively. Predawn water potential before the beginning of Phase 2 was -0.85 for plants in the water deficit treatment and -0.16 MPa for plants in the partially flooded and flooded treatments ($P < 0.0001$).

During the last three weeks of Phase 2, plants that were partially flooded before being subjected to water deficit in Phase 2 had lower Ψ_{midday} than plants that were flooded or partially flooded during Phase 2. In contrast, Ψ_{midday} of plants that were subjected to water deficit or flooding before water deficit was similar to that of plants that were flooded or

partially flooded during Phase 2. At the end of the experiment, Ψ_{midday} for plants treated with partial flooding before water deficit was -1.53 MPa, whereas Ψ_{midday} of all other treatments averaged -0.82 MPa ($P = 0.0009$). At this time, $\Psi_{predawn}$ was similar for plants in all treatments, but plants from Illinois had lower $\Psi_{predawn}$ (-0.34 MPa) than plants from Florida (-0.24 MPa; $P = 0.0278$).

Gas exchange. Photosynthesis and g_s during Phase 2 were influenced by Phase 1 treatment, but no differences were seen among provenances. Figure 4 shows P_n values immediately before and at the end of Phase 2. Before initiation of Phase 2 treatments, P_n of plants in the flood and water deficit treatments was reduced by 50 and 77%, respectively, compared with plants that were partially flooded. Throughout Phase 2, P_n of plants in the partial flood-water deficit treatment was less than that of the control, whereas plants pretreated with water deficit or flooding maintained rates of P_n similar to that of the control (Figure 4).

Patterns in g_s during Phase 1 were similar to those in Expt. 1. Figure 5 shows trends for g_s immediately before and during Phase 2. At the end of Phase 1, g_s values for plants in the flood and water deficit treatments were 61 and 33% the value of the control, respectively. Plants pretreated with flooding or water deficit during Phase 2 had similar g_s 4 d after Phase 2 treatments began irrespective of Phase 1 treatment, but g_s of plants pretreated with water deficit prior to partial flooding took 8 d to recover to a value similar to that of the control. At the end of the experiment, no differences were seen among plants in the partial flood treatment, but g_s of plants that were continually flooded was more than double that of plants that were pretreated with water deficit or partial flooding before flooding (Figure 5).

Differences between plants pretreated with flooding and partial flooding were seen in stomatal response to declining medium moisture. Conductance values for plants pretreated with partial flooding were twice the mean value of seedlings pretreated with flooding at a medium moisture of 0.50 $m^3 \cdot m^{-3}$ (container capacity) and declined rapidly as medium dried (Figure 6). In contrast, g_s of individual plants pretreated with flooding was not correlated with medium moisture (for individual plants average $R^2 = 0.19$, $P > 0.30$), but for all plants, g_s showed a slight increase with decreasing medium moisture (Figure 6). Medium moisture declined to prescribed levels for plants pretreated with partial flooding within 11 d, whereas medium moisture for plants pretreated with flooding took 16 d to reach the prescribed moisture for the water deficit treatment.

Biomass, leaf area, and visual observations. Biomass and leaf area of plants from all provenances were affected similarly by treatments, and Phase 1 treatment did not affect response to Phase 2 treatment. Thus, values presented for these variables are main effects for Phase 1 treatment, Phase 2 treatment, and provenance.

Plants subjected to water deficit during Phase 1 produced 80% less total dry biomass and 78% less leaf area than plants that were partially flooded (Table 4). Flooding also reduced total biomass and leaf area compared with plants that were partially flooded, but only by 57% and 52%, respectively. Trends were similar during Phase 2; plants treated with water deficit produced 30% less total biomass and 47% less leaf area than those in the partial flood treatment (Table 4). Flooding during Phase 2 reduced leaf area by 27% compared with the partial flood treatment, but the plants in the two treatments had similar root and shoot mass (Table 4).

Although pretreatment did not affect biomass or leaf area values, it did affect the visual quality of the plants. For plants that were exposed to water deficit during Phase 2, 22% of those that were pretreated with partial flooding had severe tip dieback (>10 cm), and 55% had noticeable leaf scorch, whereas these symptoms were not observed on any of the plants pretreated with flooding (Figure 7).

Differences were evident in biomass partitioning among provenances. Above-ground biomass of plants from Florida was similar to that of plants from South Carolina, but plants from Florida produced comparatively less root biomass. Both above- and below-ground mass was smaller for plants from Illinois than for plants from South Carolina, but both provenances had a 41% larger root:shoot ratio than plants from Florida.

Aerenchyma production. Aerenchyma was visible in all sampled roots. Root-zone moisture content during Phase 1 did not affect aerenchyma production during Phase 2. During both phases, plants that were flooded produced more aerenchyma than did those that were treated with water deficit (Table 4). During Phase 2, plants that were partially flooded also produced more aerenchyma than plants in the water deficit treatment (Table 4). Aerenchyma production was similar for all provenances.

Mortality. Fifty-five percent of plants subjected to continuous water deficit and 11% of plants treated with water deficit before flooding died before the experiment concluded. Of the plants that perished during the experiment, 50% were from Florida, 33% were from South Carolina, and 17% were from Illinois.

Discussion

Growth responses of *N. aquatica* and *N. ogeche* were not influenced by root-zone soil moisture preceding flood or water deficit. Plants subjected to water deficit showed severe

reductions in growth irrespective of when water stress was applied. Reductions in growth for plants exposed to water stress in our studies were comparable to those reported by Dickson et al. (1965) for seedlings grown at wilting point. Plants that were flooded also showed reductions in growth compared with the plants in the partially flooded control, similar to what has been seen when seedlings of *N. aquatica* are grown in stagnant water (Harms, 1973). In both experiments, reductions in growth were larger when plants were subjected to water deficit than when subjected to flooding. Even in the water deficit treatment, however, plants grew relatively quickly; all were greater than 0.45 m tall at the end of the season. However, the leaf abscission we observed in response water deficit may limit the use of this species in urban landscapes with continuously dry soils.

Seedlings of *N. ogeche* also tolerated fluctuations in soil moisture but were sensitive to water deficit as indicated by high mortality rates. Seedlings of *N. ogeche* have been cultivated successfully in drained soils and may have better tolerance to water deficit in a field or landscape setting (Williams et al., 1995) than when confined to containers.

In both experiments, growth of *N. aquatica* was influenced more substantially when stress was applied early in the season than later in the season. Seedlings of *N. aquatica* had rapid stem growth early in the summer followed by a period of slowed growth. This characteristic, which has been noted for seedlings of *N. aquatica* previously (Harms, 1973; McKelvin et al., 1995), allows trees to avoid complete submergence in wetlands that experience late-season flooding (Eyde, 1997). For both species, we saw mortality only for plants that were subjected to water deficit early in the season, indicating that water deficit may be particularly detrimental during bursts of shoot growth.

Physiological responses of *N. aquatica* to water deficit were affected by the root-zone soil moisture preceding water deficit. Plants that had optimal soil moisture for growth (partial flooding) early in the season manifested severe physiological stress when exposed to water deficit. At the end of a six-week period of water deficit, P_n of plants pretreated with partial flooding was reduced compared with plants that were subjected to continuous water deficit, but g_s for the two groups was similar. Conversely, plants that were pretreated with flooding maintained P_n similar to that of partially flooded controls, indicating that preconditioning with flooding may help sustain photosynthetic response during subsequent water deficit. Plants pretreated with flooding had smaller total biomass and smaller leaf area than plants that were partially flooded, and medium moisture for these plants did not decrease as rapidly as for those that were pretreated with partial flooding upon initial exposure to water deficit.

Plants pretreated with partial flooding before being exposed to water deficit also had reduced visual appeal compared with those pretreated with flooding. The negative response of plants pretreated with partial flooding as opposed to responses of plants pretreated with flooding may have been due in part to the larger size and increased transpirational demand of the trees when water deficit was imposed. The larger trees may also have had a more rapid rate of soil drying than the smaller plants. Plants exposed to gradual soil drying can often adapt to low water potentials better than those that are desiccated rapidly (ZhouPing et al., 1999).

The sequence of flooding and water deficit did not affect trends in biomass, height growth, gas exchange, aerenchyma production, or water potential, although it did affect the intensity of the responses. This is in contrast with the Everglade tree species studied by Miao

et al. (2009), which showed greater reductions in biomass and survival when seedlings were subjected to flooding before water deficit. However, our results are consistent with studies of *Taxodium distichum* L. (bald cypress) that show that pretreatment with flooding does not alter subsequent g_s or P_n during mild drought (Elcan and Pezeshki, 2002).

Seedlings from the continually flooded site in Florida developed greater root mass in response to continual flooding than seedlings from the periodically flooded site in Illinois and had a smaller root:shoot ratio than plants from either Illinois or South Carolina. In addition, seedlings from Florida formed more total biomass than seedlings from Illinois. Although seedlings from Florida had greater productivity when water was limiting than seedlings from Illinois, responses to water deficit and fluctuations in soil moisture were similar for all provenances. Plants from northern latitudes that maintain smaller biomass may be better suited to urban landscapes because reductions in leaf area and size are not as substantial upon exposure to water deficit as for plants from southern latitudes.

Nyssa aquatica and *N. ogeche* could be valuable additions to the selection of urban trees that can withstand flooding as well as short episodes of water deficit. However, the use of both species may be limited in continuously dry soils. In a production setting, pretreating seedlings of *N. aquatica* with flooding early in the season or before transplanting could reduce stress in future water deficit and improve ornamental appeal by slowing growth and preventing significant leaf drop and stem dieback when water deficit occurs later in the season.

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Table 1. Predawn leaf water potential ($\Psi_{predawn}$) of seedlings of *N. aquatica* from Florida and Illinois and *N. ogeche*. Measurements were taken on 21 Oct. 2006 after seedlings had been subjected to 53 d of Phase 1 treatment and 59 d of Phase 2 treatment. Variables were similar for both provenances and species, so values are means of the three groups. Different letters within a column indicate differences at $P < 0.05$ according to the Ryan-Einot-Gabriel-Welsch multiple range test.

Phase 1	Phase 2	n	$\Psi_{predawn}$ (MPa)
Partial flood	Partial flood	13	-0.38 a
Flood	Flood	15	-0.44 ab
Water deficit	Water deficit	9	-0.44 ab
Partial flood	Water deficit	11	-0.61 b
Water deficit	Flood	12	-0.43 a
Flood	Water deficit	12	-0.43 ab

Table 2. Dry weights, leaf area, and average leaf size of seedlings of *Nyssa aquatica* and *Nyssa ogeche*. Seedlings were grown in 1.8-L pots in a glass-glazed greenhouse and were harvested after being subjected to 53 d of Phase 1 treatment and 59 d of Phase 2 treatment. Values represent means of n replicates, where n represents the number of seedlings living at the end of Phase 2. Seedlings of *N. aquatica* from Florida and Illinois had similar responses to treatments, so values given are main effects of provenance and treatment. For main effects of provenance and treatment for each species, different letters within a column indicate differences at $P < 0.05$ according to the Ryan-Einot-Gabriel-Welsch multiple range test.

Species	Provenance	Phase 1	Phase 2	n	Total biomass (g)	Stem (g)	Leaf (g)	Leaf area (m^2)	Leaf size (cm^2)
N. aquatica	Florida			30	52 a	29 a	9 a	0.23 a	80 a
	Illinois			28	37 b	20 b	7 b	0.20 a	59 b
N. aquatica		Partial flood	Partial flood	10	71 a	41 a	12 a	0.33 a	87 a
		Flood	Flood	10	52 b	29 b	8 b	0.24 b	72 ab
		Water deficit	Water deficit	9	33 c	17 c	7 bc	0.18 b	51 b
		Partial flood	Water deficit	10	47 bc	27 b	8 bc	0.19 b	85 a
		Flood	Water deficit	10	31 c	17 c	5 c	0.17 b	58 b
		Water deficit	Flood	9	31 c	17 c	6 b	0.19 b	67 ab

Table 2. (continued)

Provenance Species	Phase 1	Phase 2	n	Total biomass (g)	Stem (g)	Leaf (g)	Leaf area (m^2)	Leaf size (cm^2)
N. ogeche	Partial flood	Partial flood	5	57 a	29 a	15 a	0.31 a	21 a
	Flood	Flood	5	39 ab	21 a	11 ab	0.30 a	26 a
	Water deficit	Water deficit	2	16 b	6 b	6 b	0.17 a	24 a
	Partial flood	Water deficit	5	35 b	16 ab	10 ab	0.22 a	16 a
	Flood	Water deficit	5	31 b	21 ab	9 ab	0.19 a	18 a
	Water deficit	Flood	4	32 ab	16 ab	9 ab	0.26 a	24 a

Table 3. Dry root mass (g) of seedlings of *Nyssa aquatica* from Florida and Illinois. Seedlings in their first year of growth were subjected to two consecutive phases in which soil moisture was manipulated; the first phase lasted 53 d, and the second phase lasted 59 d. Values are means of five replicates. For each provenance, different letters within a column indicate differences at $P < 0.05$ according to the Ryan-Einot-Gabriel-Welsch multiple range test.

Phase 1	Phase 2	Florida	Illinois
Partial flood	Partial flood	22 a	16 a
Flood	Flood	18 ab	9 abc
Water deficit	Water deficit	11 c	7 bc
Partial flood	Water deficit	14 bc	12 ab
Flood	Water deficit	8 c	8 bc
Water deficit	Flood	11 c	4 c

Table 4. Dry weights, root:shoot ratio, total leaf area, and percentage aerenchyma in the root cortex for *N. aquatica* from Florida, Illinois, and South Carolina. Plants were cuttings rooted the previous year and grown in a glass-glazed greenhouse. Each plant was subjected to two consecutive soil moisture regimes (Phase 1 followed by Phase 2), each of which lasted approximately six weeks. Percentage aerenchyma was calculated from cross sections of 1- to 3-mm diameter roots 10-cm from the root tip from each plant. Values are means of 27 replications. For Phase 1, Phase 2, and provenance, different letters within a column indicate differences at $P < 0.05$ according to Tukey's studentized range test.

	Root (g)	Stem (g)	Leaf (g)	Root: shoot	Leaf area (m^2)	Aerenchyma in cortex (%)
Phase 1						
Water deficit	4 c	8 c	3 c	0.48 a	0.07 c	15 b
Flood	10 b	15 b	8 b	0.48 a	0.17 b	19 a
Partial flood	20 a	38 a	18 a	0.38 b	0.34 a	15 ab
Phase 2						
Water deficit	10 b	16 b	7 c	0.48 a	0.14 c	11 b
Flood	12 a	21 ab	10 b	0.47 ab	0.20 b	19 a
Partial flood	12 a	23 a	13 a	0.39 b	0.27 a	18 a
Provenance						
Florida	11 b	24 a	12 ab	0.35 b	0.24 a	16 a
Illinois	10 b	15 b	6 b	0.53 a	0.12 b	14 a
South Carolina	13 a	21 a	12 a	0.46 a	0.26 a	17 a

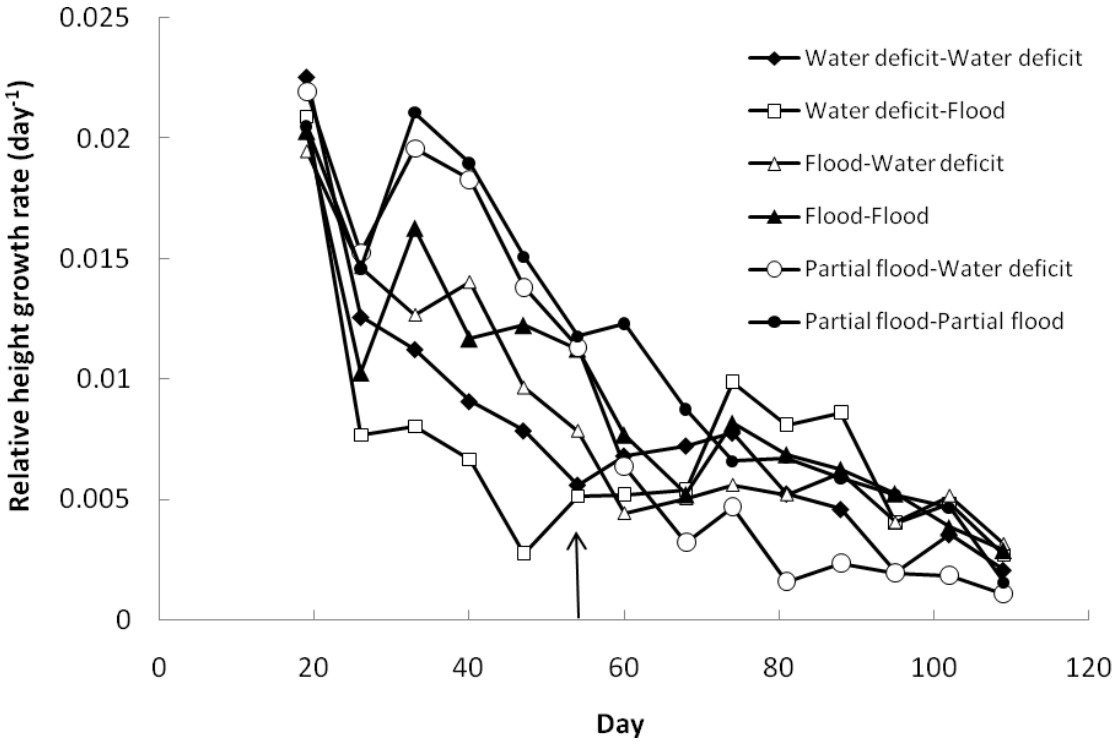

Figure 1. Relative height growth rate (d^{-1}) for seedlings of *N. aquatica* during Expt. 1. Seedlings of both provenances had similar relative height growth rates, so values are means of five replicates from each provenance. The arrow indicates the date Phase 1 ended and Phase 2 began.

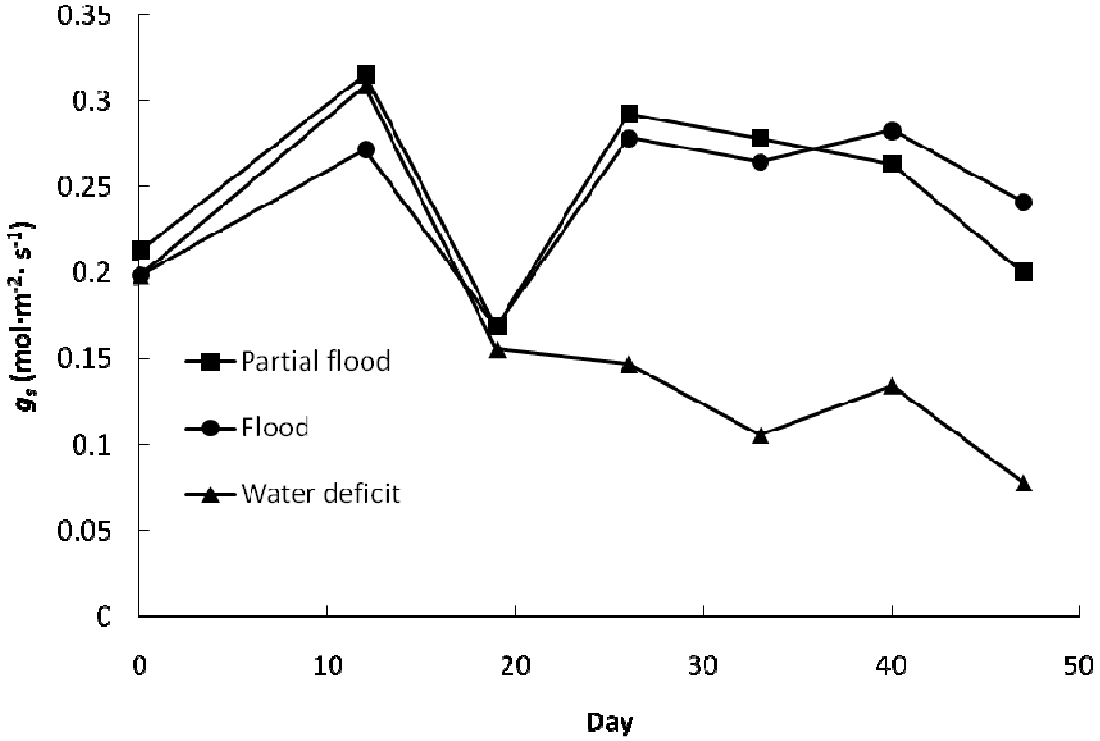

Figure 2. Stomatal conductance (g_s) of seedlings of *N. aquatica* from Florida and Illinois and *N. ogeche* that were subjected to partial flooding, flooding, or water deficit during Phase 1 of Expt. 1. Conductance values of both species and provenances of *N. aquatica* were similar, and values are means of 30 plants, 10 replicates from each species or provenance.

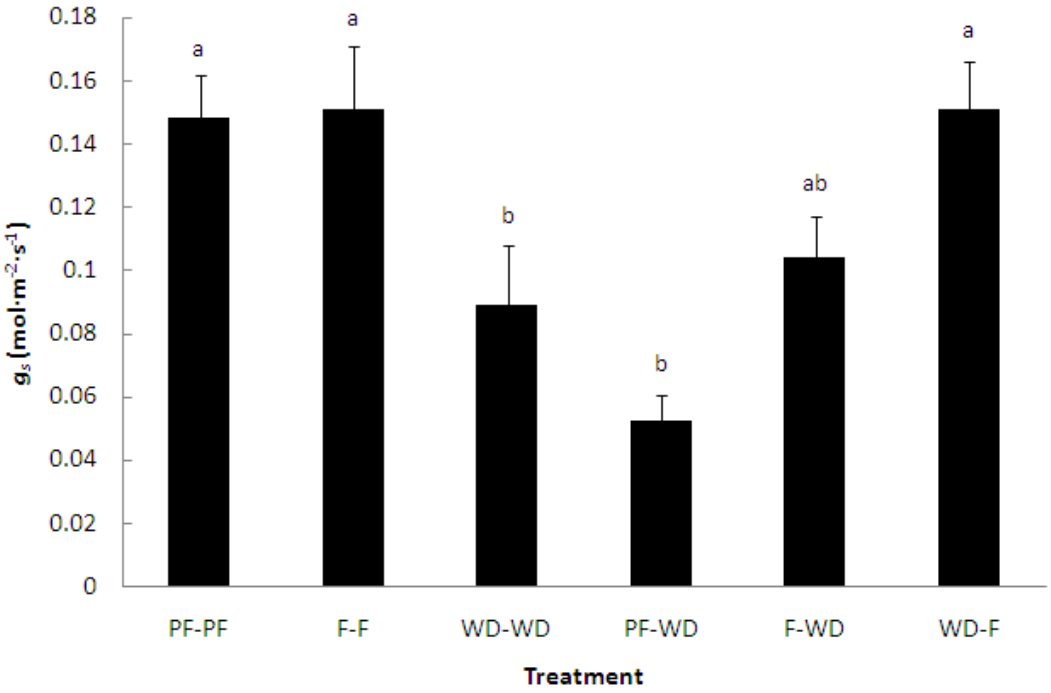

Figure 3. Mean stomatal conductance (g_s) for seedlings of *Nyssa ogeche* and *Nyssa aquatica* from Florida and Illinois. Measurements were taken at midday from the newest fully expanded leaf of each seedling on 27 Sept. 2006, 34 d after Phase 2 began. Environmental conditions at midday were: *PAR* = 800 mol·m^{-2}·s^{-1}, RH = 39%, and air temperature = 21° C. Treatments: PF = Partial flood, F = Flood, WD = Water deficit. Error bars represent standard errors.

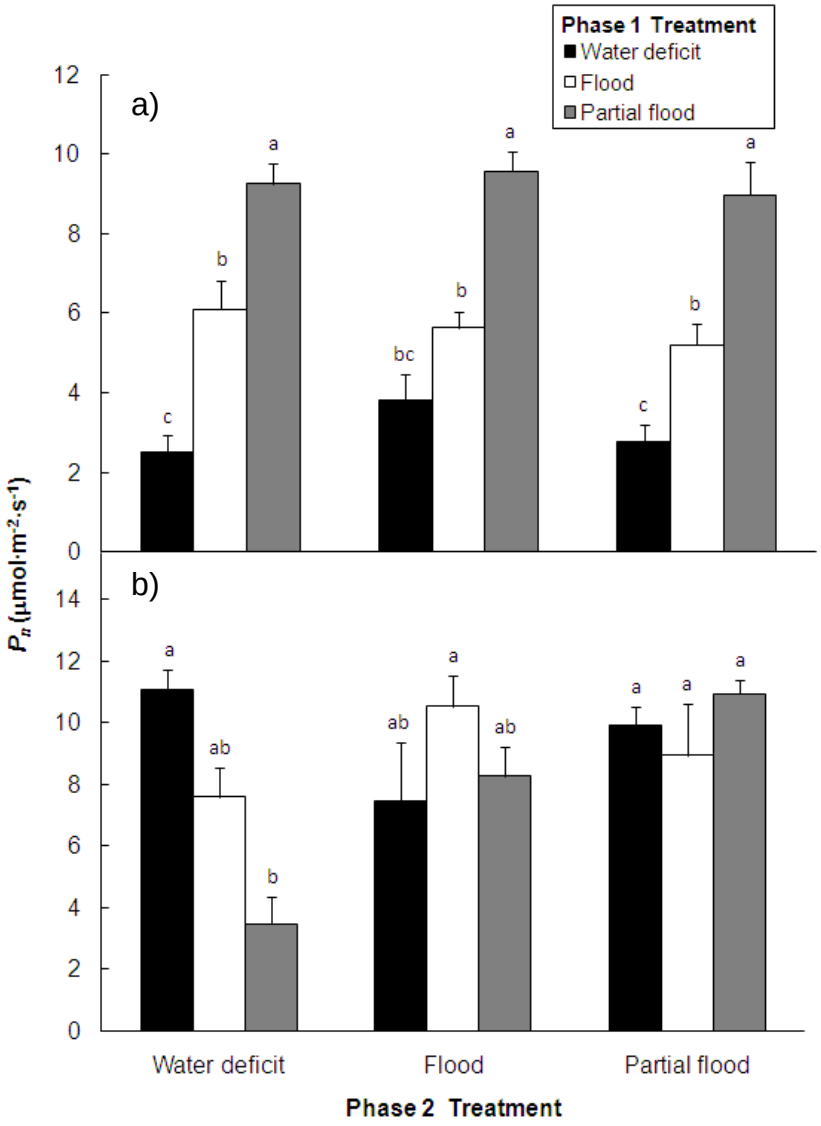

Figure 4. Photosynthesis of *N. aquatica* 4 d before Phase 2 began (a) and at the end of Phase 2 (b). Plants were grown from stem cuttings rooted the previous year, and measurements of P_n were taken on the newest fully expanded leaf of each plant at midday. For plants treated with Phase 2 water deficit, those pretreated with water deficit during Phase 1 maintained higher P_n than those pretreated with partial flood (control) beginning one week after the Phase 2 treatments were applied. Different letters indicate differences at $P < 0.05$ according to Tukey's multiple range test.

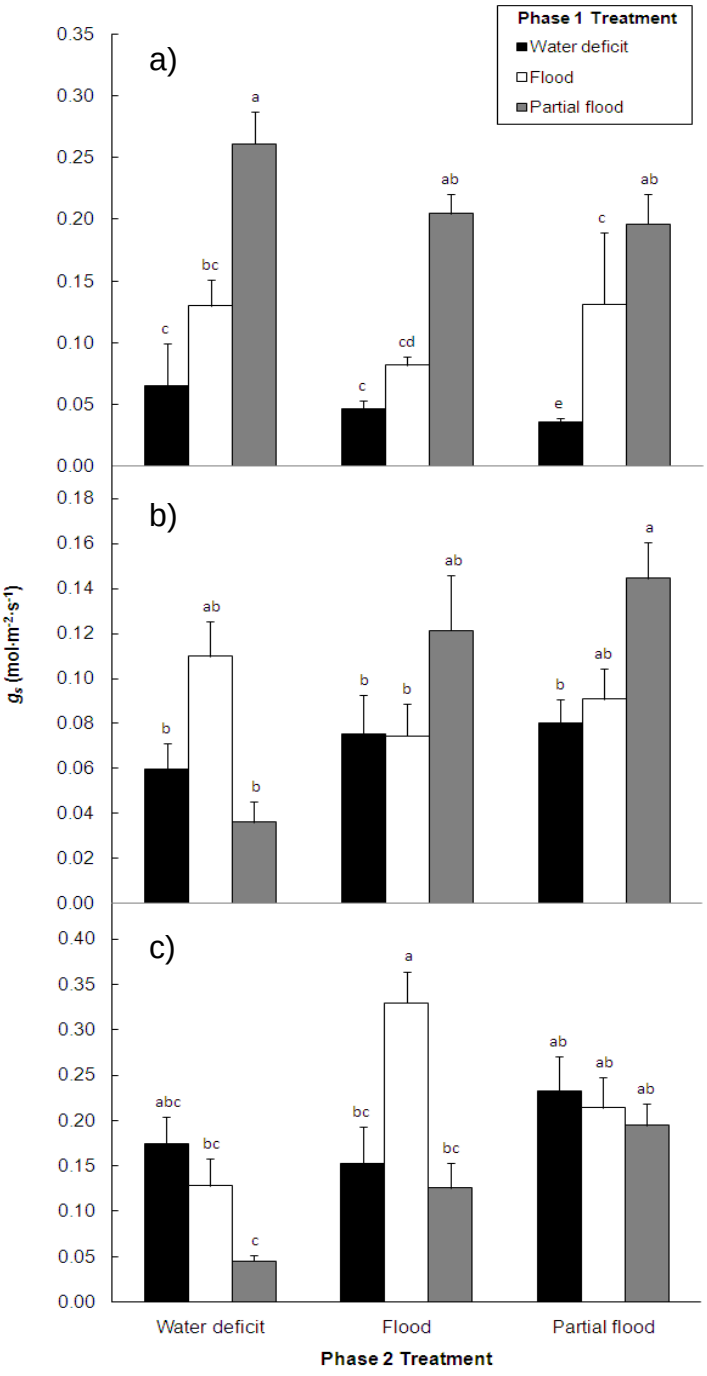

Figure 5. Stomatal conductance (g_s) of *N. aquatica* 4 d before the initiation of Phase 2 (a), 4 d after Phase 2 began (b), and on the last day of the experiment (c). Plants were grown in a

glass-glazed greenhouse from stem cuttings rooted the previous year, and measurements of g_s were taken on the newest fully expanded leaf of each plant at midday. Different letters indicate differences at $P < 0.05$ according to Tukey's multiple range test.

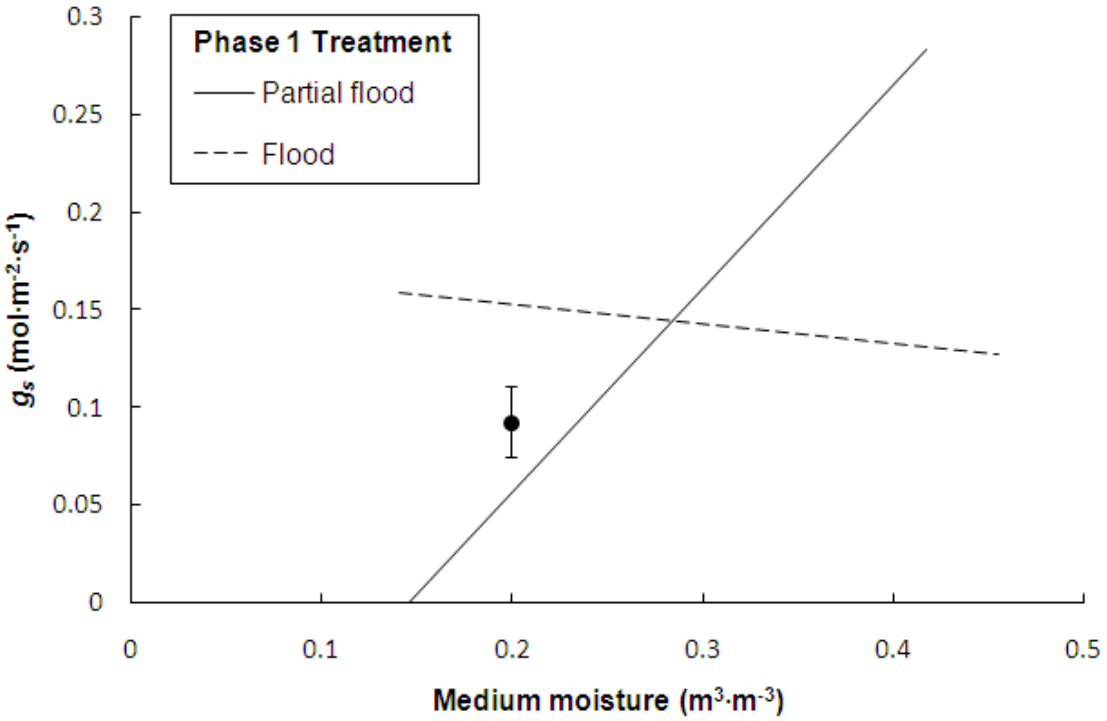

Figure 6. Relationship between g_s and medium moisture for *N. aquatica* pretreated with partial flooding (solid line) or flooding (dashed line) for six weeks before being subjected to water deficit during Phase 2. The closed circle represents mean g_s ($\pm$ standard error) for plants that were subjected to water deficit during both phases. Lines are means of regressions calculated for individual plants during the first 16 d of Phase 2. Partial flood: g_s = 1.043 (medium moisture) – 0.151; Flood: g_s = -0.100 (medium moisture) + 0.173. Slopes of the two lines are different at $P < 0.0001$.

Figure 7. *Nyssa aquatica* trees pretreated with water deficit (a) flooding (b) and partial flooding (c) during Phase 1 and exposed to water deficit during Phase 2 of Expt. 2. Some trees pretreated with partial flooding had severe tip dieback and leaf scorch, whereas none of the trees pretreated with flooding displayed these symptoms.

CHAPTER 3. RESPONSES OF THREE NORTH AMERICAN *NYSSA* SPECIES TO WATER DEFICIT

A manuscript to be submitted to Castanea

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Abstract. We examined responses to water deficit for seedlings of three wetland tree species native to the eastern United States: *Nyssa aquatica* (water tupelo), *Nyssa ogeche* (Ogeechee tupelo), and *Nyssa sylvatica* (black gum). Seedlings were grown in soilless medium in 7.6-L containers in a glass-glazed greenhouse, and were subjected to either water deficit (medium dried to -0.5 to -1.0 MPa before irrigating to container capacity) or partially saturated medium. Nineteen percent of the *N. ogeche* seedlings died within 63 days of being subjected to water deficit, but no mortality was seen for either of the other two species during this time. All seedlings subjected to water deficit reduced biomass, photosynthesis, and stomatal conductance compared with controls. However, only *N. aquatica* increased root:shoot ratio in water deficit. Seedlings of *N. sylvatica* grew more slowly and used water more conservatively than the other two species. The large biomass produced by seedlings of *N. ogeche* along with its inability to adjust root:shoot ratio in water deficit may have contributed to the relative sensitivity of this species to water deficit.

Introduction

Populations of wetland trees in the southeastern United States have been declining for several centuries due to urbanization and diversion of water for agricultural and municipal use (King et al., 2009; Stallins et al., 2009). For *Nyssa ogeche* Bartram ex. Marsh.

(Ogeechee tupelo), a small tree native to southern Georgia, northern Florida, and several counties in Alabama and South Carolina, declines of more than 40% have been observed in the last 30 years in the Apalachicola River Basin (Stallins et al., 2009). Reductions in population density may be associated with drying of soils in this region (Stallins et al., 2009). In the wild, *Nyssa ogeche* is restricted to soils that are wet continuously (Kossuth and Scheer, 1990). However, we know of no published studies that examine the whole-plant or physiological effects of water deficit on *N. ogeche*.

Two other *Nyssa* species, *Nyssa sylvatica* Marsh. (black gum) and *Nyssa aquatica* L. (water tupelo), also inhabit wetlands in the southeastern United States. In the wild, *N. sylvatica* grows in alluvial stream bottoms and uplands (McGee, 1990), and *N. aquatica* inhabits periodically and continuously flooded soils in the Lower Mississippi River Valley and Atlantic and Gulf coastal plains (Johnson, 1990). *Nyssa aquatica* and *N. sylvatica* are considered sensitive to water deficit compared with mesic trees but have adaptations for tolerating periods of water deficit (Augé et al., 2002; Dickson et al., 1965; Dickson and Broyer, 1972; Maherali et al., 2004).

The objective of this experiment was to compare the responses of seedlings of *N. ogeche*, *N. aquatica*, and *N. sylvatica* to water deficit. Specifically, we were interested in determining how biomass accumulation and partitioning, leaf area, and gas exchange were affected by water deficit.

Materials and Methods

Plant material. Seedlings of *N. aquatica* were grown from seeds collected in 2006 from Union County, IL, Aiken County, SC, and Levy County, FL. Seedlings of *N. ogeche* were grown from seeds collected in 2006 in Liberty County, FL. Seeds of both species were

overwintered in a minimally heated greenhouse and germinated in the spring of 2007. Seedlings of *N. sylvatica* were grown from seeds collected at the Minnesota Landscape Arboretum (Chanhassen, MN) from an open-pollinated tree that arboretum records show originated from the wild in Lincoln Co., Missouri. Seedlings of *Nyssa sylvatica* were germinated in the spring of 2005 and grown in 2.5-L square containers (10 cm diameter, 25 cm tall) in soilless media composed of composted bark, peat, and coarse sand in an outdoor nursery at the Minnesota Landscape Arboretum. In June 2007, seedlings were moved to the Iowa State University Research Greenhouses (Ames, IA). All seedlings were transplanted into 7.6-L pots (21 cm diameter) in soilless medium composed of 60% processed pine bark, 30% sphagnum peat, and 10% perlite (Conrad Fafard mix #51, Agawam, MA) three weeks before treatments were initiated. Seedlings were irrigated daily with tap water and fertilized once each week with 385 mg $N \cdot L^{-1}$ Peters Excel All-Purpose 21N-2.2P-16.6K (Scotts, Marietta, GA) until treatments began. All seedlings were approximately the same height when treatments were initiated.

Treatments. Treatments began on 23 July 2007 and continued for 63 days. Eighteen seedlings of each species and provenance were randomly assigned to receive each one of two irrigation treatments: partial flood (control) or water deficit. The control treatment was chosen based on the optimal growth achieved by partially flooded seedlings of *N. aquatica* and *N. ogeche* during previous experiments (see Chapter 2). Pots in the control treatment were set in plastic saucers (bottom diameter 20-cm bottom diameter, 9-cm tall) filled with tap water so medium in the bottom of the pot was kept saturated. Water in the saucers was replenished daily as needed. Water deficit was applied in a cyclic manner to each taxonomic

group by withholding water until the moisture content in the upper 6-cm of the medium in 10 of the 18 replications fell below 0.15 $m^3 \cdot m^{-3}$ for the first cycle of water deficit and 0.10 $m^3 \cdot m^{-3}$ for each subsequent cycle before irrigation to container capacity. Moisture release curves for the medium showed that moisture tension was approximately -0.5 MPa at 0.15 $m^3 \cdot m^{-3}$ and approximately -1.0 MPa at 0.10 $m^3 \cdot m^{-3}$. At 10 $m^3 \cdot m^{-3}$, seedlings of *N. ogeche* and *N. aquatica* wilted at midday. Medium moisture in the upper 6 cm of the medium was measured daily using a Theda probe (Decagon Devices, Pullman, WA). At each irrigation, seedlings in both the control and water deficit treatment were fertilized with 385 mg $N \cdot L^{-1}$ Peters Excel All-Purpose 21N-2.2P-16.6K (Scotts, Marietta, GA). Seedlings in the control were removed from saucers before fertilizing and allowed to drain before being placed back in the saucers.

Environmental conditions. The experiment was conducted in a glass-glazed greenhouse in Ames, IA, with no supplemental irradiation. Photosynthetically active radiation (*PAR*) at canopy level was measured weekly at midday using a Li-1600 quantum sensor (LiCor, Lincoln, NE). Average midday PAR was 457 $\pm$ 42 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Greenhouse temperature was logged hourly during the study period using a Thermochron i-Button (Dallas Semiconductor, Dallas, TX). During the experiment, temperature in the greenhouse ranged from 13 to 44 C, and mean daily minimum/maximum temperatures were 17/32 °C.

Data collection. Measurements of photosynthesis (P_n) and stomatal conductance (g_s) were taken between 1000 and 1400 HR on the youngest fully expanded leaf of each plant using a Li-6400 Photosynthesis System (Li-Cor, Lincoln, NE). Instantaneous water use efficiency (WUE_i) was calculated by dividing P_n by measurements of transpiration obtained using the Li-6400 system. Measurements were taken for all plants on days 8, 12, 19, 26, and

61. Gas exchange measurements were also taken for each taxonomic group immediately prior to watering. For all measurements, chamber conditions were: *PAR* = 600 µmol·m^{-2}·s^{-1}, CO_2 = 400 µmol·mol^{-1}, and relative humidity = 50%.

At the end of the study, three plants from each treatment were selected from each of the three species for pressure-volume analyses. Seedlings of *N. aquatica* were taken from the Florida provenance. Petioles of recently fully expanded leaves from all sampled plants were cut under deionized water with a razor blade, placed in a flask containing deionized water in a dark cooler at 4 °C, and stored for >15 hr before measurements began. Six leaves per day were sampled randomly for analysis (Cheung et al., 1975), so all measurements were completed within 3 days. Osmotic potential at full turgor (Ψ_s^{100}) and bulk modulus of elasticity were determined using the methods of Pallardy et al. (1991).

Nine seedlings from each taxonomic group and treatment were randomly selected for harvest on 24 Sept. 2007. Leaves were removed from trees and passed through a leaf area meter (Li-3100, Li-Cor, Lincoln, NE) to determine total leaf area. Leaves, shoots, and roots were dried at 70 °C for 48 hours before weighing. Specific leaf area (SLA) was calculated by dividing leaf area by total dry leaf weight. The remaining nine seedlings from each taxonomic group and treatment were overwintered in a greenhouse that was kept above 10 °C (mean daily maximum = 15 °C) and watered once a week until the following spring. Percentage survival was recorded on 8 May 2008, when leaves had emerged from all surviving seedlings.

Experimental design and statistical analyses. The experiment was a completely randomized 5 x 2 factorial with five provenance/species groups (*N. aquatica* from Florida, *N. aquatica* from Illinois, *N. aquatica* from South Carolina, *N. sylvatica*, and *N. ogeche*) and

two watering treatments (control and water deficit). Biomass, leaf area, and pressure-volume data were analyzed using a general linear model (GLM) in SAS (v.9; Cary, NC), and means were separated using the Ryan-Einot-Gabriel-Welsch multiple range test. Because of inherent differences in plant growth and response to water deficit, biomass and leaf area data from individual taxa were analyzed separately. Values of g_s, P_n, and WUE_i for plants in the control treatment were analyzed across all days using a MIXED model in SAS and treating individual plants as random variables. Values of g_s and P_n were regressed against medium moisture for each plant in the water deficit treatment, and regression coefficients were averaged for each species or provenance to create a mean regression equation. Significance of regression coefficients was tested using a general linear model. Mean separation was conducted using the Ryan-Einot-Gabriel-Welsch multiple range test. Contrasts were used to make specific comparisons of interest.

Results

Mortality. Seedlings of *N. ogeche* subjected to water deficit experienced 19% mortality by the end of the first growing season. Half of the *N. ogeche* seedlings and 13% of the *N. aquatica* seedlings from Illinois that were overwintered also died.

Biomass and leaf area. Seedlings of all species had reduced total biomass in response to repeated cycles of water deficit, but only seedlings of *N. aquatica* had altered biomass partitioning (Table 1). Averaged over provenances, root:shoot ratio for seedlings of *N. aquatica* in the water deficit treatment was 47% larger than in the control. Compared with the control, leaf area was also smaller for seedlings of *N. aquatica* and *N. ogeche* that were subjected to water deficit (Table 1).

Gas exchange. In the control treatment, seedlings of *N. aquatica* from Illinois had 33% and 12% greater g_s and P_n, respectively, but 16% smaller WUE_i than seedlings from Florida (Table 2). Stomatal conductance and P_n of seedlings of *N. sylvatica* were smaller than that of the other two species. Seedlings of *N. ogeche* had g_s similar to that of all *N. aquatica* seedlings, but P_n was smaller than that of *N. aquatica* from Illinois and South Carolina provenances.

During water deficit, seedlings of *N. aquatica* from Florida and *N. ogeche* used water more quickly than the other species and provenances. Seedlings of these two taxa completed the first water deficit cycle in 12 days, whereas seedlings of *N. aquatica* from Illinois and *N. sylvatica* took 19 days to complete the first cycle (Table 3). Because of the comparatively fast initial water-use rate, seedlings of *N. aquatica* from Florida and *N. ogeche* finished one more water deficit cycle than the other seedlings in the study.

Stomatal conductance for all seedlings in the water deficit treatment decreased linearly as medium moisture decreased (Figure 1). Seedlings of *N. sylvatica* maintained lower g_s across the range of medium moisture sampled (0.05 to 0.50 $m^3 \cdot m^{-3}$) than seedlings of the other two species. Conversely, g_s of seedlings of *N. aquatica* from Illinois was greater than that of the other species and provenances in wet medium but decreased more rapidly as medium moisture decreased (Figure 1). A similar trend among species and provenances was seen for P_n, which decreased logarithmically with medium moisture (Figure 1).

Data from pressure-volume analyses are shown in Table 4. Because sample sizes were small, our power to detect differences was limited, and no differences were detected at $P < 0.05$. However, values of ε were larger for all species in the control than in the water deficit treatment ($P = 0.0941$). In addition, osmotic potential for seedlings of *N. aquatica*

and *N. ogeche* subjected to water deficit was more negative than for the controls (P = 0.0669).

Discussion

High mortality of seedlings of *N. ogeche* indicates that this species is more sensitive to water deficit than *N. aquatica* and *N. sylvatica*. Biomass partitioning may have contributed to the relative sensitivity of *N. ogeche* to the imposed water deficit. Seedlings of *N. ogeche* had small root:shoot in both treatments, whereas seedlings of *N. sylvatica* maintained a relatively large root:shoot in both treatments. In other studies, seedlings of *N. sylvatica* have been shown to decrease root:shoot when subjected to water deficit as well as excess water compared with well-watered, but drained, plants (Nash and Graves, 1993). Of the three species, only *N. aquatica* increased root:shoot in response to water deficit. The ability of seedlings of *N. aquatica* to adjust root:shoot in response to drying soils may give this species an advantage over seedlings of the other two *Nyssa* species by allowing extraction of water from larger volumes of soil and may be of greater significance when rooting volume is not constrained.

Another factor that may have contributed to the relative success of seedlings of *N. sylvatica* in water deficit was the maintenance of a small leaf area. In the control, seedlings of *N. ogeche* and *N. aquatica* had larger total biomass and leaf area at the end of the growing season than *N. sylvatica* despite the fact that seedlings of *N. sylvatica* were one year older and were of similar size to the other species at the beginning of the study. Seedlings of *N. aquatica* from Illinois, which were shorter than those from Florida and South Carolina, maintained a larger leaf area and experienced a smaller reduction in leaf area than seedlings from the other two provenances in water deficit.

In both treatments, g_s and P_n than were larger per unit leaf area for seedlings of *N. ogeche* and *N. aquatica* than for seedlings of *N. sylvatica*. Greater conductance rates of *N. ogeche* and *N. aquatica* combined with large leaf area may have increased the relative susceptibility of these two species to water deficit. Seedlings of *N. aquatica* from Illinois had greater g_s and P_n than those from Florida or South Carolina when medium moisture was above 0.15 $m^3 \cdot m^{-3}$. Seedlings of *Picea mariana* (Mill) B.S.P. (black spruce) from northern sources also had greater P_n than seedlings from southern sources early in the season, but showed an earlier autumnal decline (Johnson et al., 1996).

All plants have the potential to reduce cellular elasticity under water deficit. Although this strategy does not help maintain growth, it may help maintain relative water content of tissues and protect cells from increasing concentrations of potentially toxic ions (Pallardy, 2008). In addition, seedlings of *N. aquatica* and *N. ogeche* may have capacity for osmotic adjustment, which can help maintain leaf water potential in drying soils and may be indicative of drought acclimation (Wikberg and Ögren, 2007).

Summary

All of the *Nyssa* species used in this study possessed mechanisms to tolerate water deficit. However, seedlings of *N. ogeche* were more sensitive to the imposed water deficit than the other species. Sensitivity to water deficit for seedlings of this species may be associated with its propensity to produce a large amount of biomass when water is not limiting and its inability to adjust root:shoot ratio when water is limiting.

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Table 1. Total dry biomass, root:shoot ratio, and total leaf area of seedlings of *N. aquatica*, *N. ogeche*, and *N. sylvatica* exposed to 63 days of water deficit or partial flooding (control). One- and two-year-old seedlings were grown in 7.6-L pots in a glass-glazed greenhouse. Water deficit was applied in a cyclical manner by withholding water from each species-provenance group until medium moisture in the upper 6 cm of the pot fell below 0.15 $m^3 \cdot m^{-3}$ for 10 of 18 replicates. At the end of the study half of the plants were randomly selected for harvesting, so values represent means of 9 replicates. Within a column, asterisks denote differences between seedlings in the partial flood and water deficit treatments at $P < 0.0001$ (***), $P < 0.01$ (**), and $P < 0.05$ (*).

Species	Provenance	Treatment	Total dry biomass (g)	Root: shoot	Leaf area (m^2)
N. aquatica	Florida	Partial flood	99***	0.39***	0.39***
		Water deficit	31	0.70	0.07
	Illinois	Partial flood	55**	0.52**	0.22**
		Water deficit	28	0.67	0.09
	South Carolina	Partial flood	68***	0.45**	0.27***
		Water deficit	25	0.62	0.06
N. ogeche		Partial flood	121***	0.30	0.68***
		Water deficit	24	0.37	0.10

Table 1. (continued)

Species	Provenance	Treatment	Total dry biomass (g)	Root: shoot	Leaf area (m^2)
N. sylvatica		Partial flood	37*	0.70	0.21
		Water deficit	24	0.67	0.14

Table 2. Stomatal conductance, photosynthesis, and instantaneous water use efficiency for the control seedlings. Values for gas exchange parameters are means of data taken on days 1, 8, 12, 15, 40, 47, and 61. Different letters within a column represent differences at $P < 0.05$ according to the Ryan-Einot-Gabriel-Welsch multiple range test.

Species	Provenance	g_s ($mol \cdot m^{-2} \cdot s^{-1}$)	P_n ($\mu mol \cdot m^{-2} \cdot s^{-1}$)	WUE_i ($\mu mol\ CO_2 \cdot mol\ H_2O^{-1}$)
N. aquatica	Florida	0.21 b	8.65 bc	2.80 a
	Illinois	0.28 a	9.73 a	2.34 b
	South Carolina	0.25 ab	9.40 ab	2.73 ab
N. ogeche		0.25 ab	7.75 c	2.34 ab
N. sylvatica		0.13 c	4.87 d	2.29 ab

Table 3. Number of days between watering cycles and mean medium moisture (± standard error) on the last day of each watering cycle. Soil moisture was measured in the upper 6 cm of the medium, and seedlings within each group were irrigated to container capacity when medium moisture fell below 0.15 $m^3 \cdot m^{-3}$ for 10 of 18 replicates. Seedlings were in their first year of growth and were grown in 7.6-L pots in a glass-glazed greenhouse.

Species	Provenance	Cycle		
		1	2	3
		Days of drying		
N. aquatica	Florida	12	20	21
	Illinois	19	21	—
	South Carolina	16	22	—
N. ogeche		12	18	19
N. sylvatica		19	25	—
		Mean medium moisture before watering ($m^3 \cdot m^{-3}$)		
N. aquatica	Florida	0.124 ± 0.004	0.070 ± 0.002	0.072 ± 0.004
	Illinois	0.146 ± 0.007	0.098 ± 0.016	—
	South Carolina	0.192 ± 0.088	0.083 ± 0.005	—
N. ogeche		0.113 ± 0.75	0.078 ± 0.003	0.076 ± 0.004
N. sylvatica		0.153 ± 0.010	0.086 ± 0.006	—

Table 4. Water potential at full turgor (Ψ), osmotic potential at full turgor (Ψ_s^{100}), and bulk modulus of elasticity (ε) for leaves of seedlings that were subjected to 63 days of cyclic water deficit or partial flooding. Seedlings of *N. aquatica* were grown from seeds collected in Levy Co., Florida. Values are means of three replicates. No significant differences were found among species or treatments for any of the variables.

Species	Treatment	Ψ (MPa)	Ψ_s (MPa)	ε
N. aquatica	Water deficit	-0.02	-0.63	12.2
	Partial flood	-0.06	-0.57	20.1
N. ogeche	Water deficit	-0.03	-0.68	8.6
	Partial flood	-0.01	-0.56	14.8
N. sylvatica	Water deficit	-0.06	-0.86	11.3
	Partial flood	-0.06	-0.86	12.1

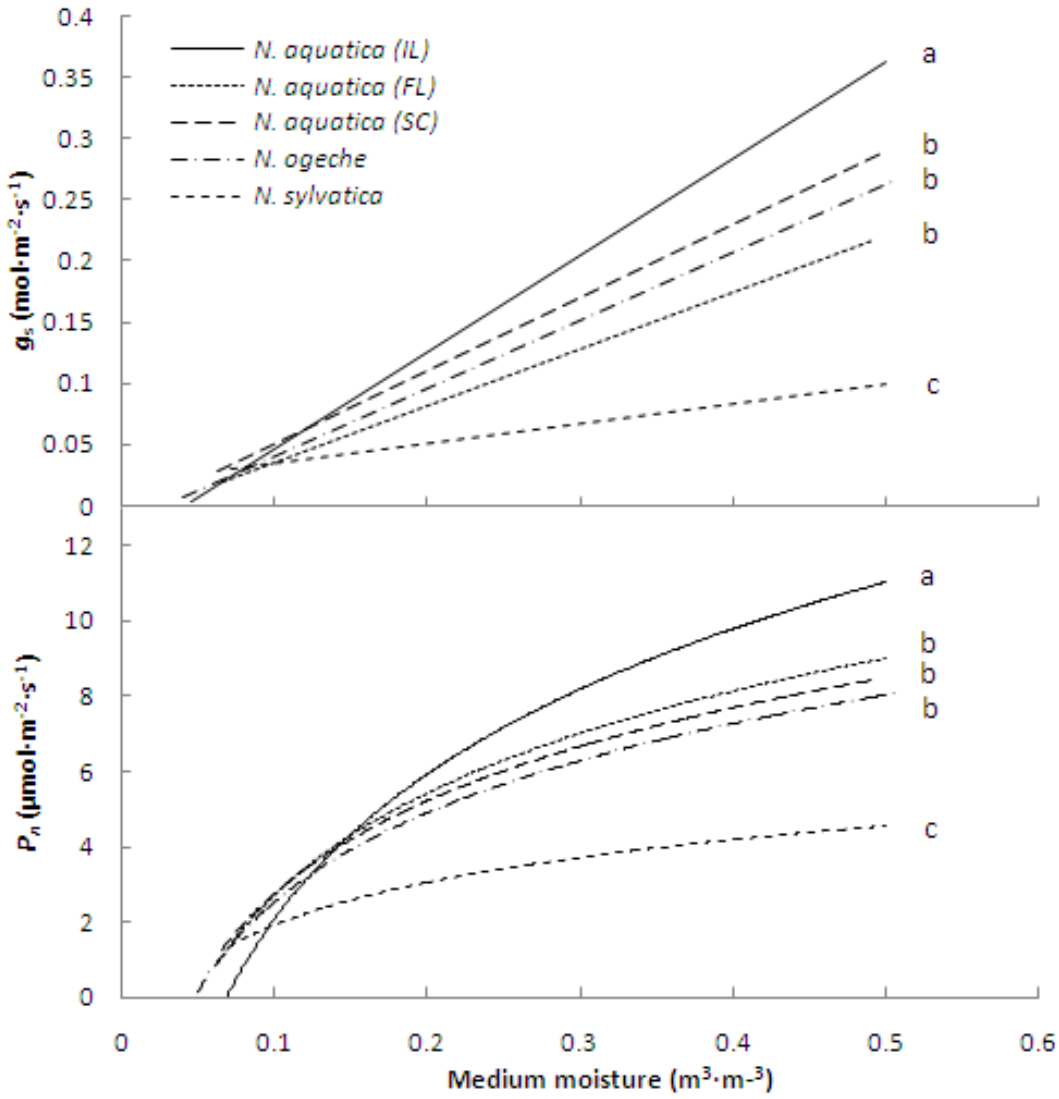

Figure 1. Stomatal conductance (g_s) and photosynthesis (P_n) as a function of medium moisture. Regression curves are means of curves generated for individual seedlings. Slopes of regression curves followed by different letters are different at $P \leq 0.05$ according to the Ryan-Einot-Gabriel-Welsch multiple range test. Regression equations: *N. aquatica* (IL), g_s = 0.79(medium moisture) – 0.03; *N. aquatica* (FL) g_s = 0.47(medium moisture) – 0.01; *N. aquatica* (SC), g_s = 0.60(medium moisture) – 0.01; *N. ogeche*, g_s = 0.56(medium moisture) – 0.02; *N. sylvatica*, g_s = 0.16(medium moisture) – 0.02; *N. aquatica* (IL), P_n = [5.58 *

ln(medium moisture)] + 14.92; *N. aquatica* (FL) P_n = [3.92 * ln(medium moisture)] + 11.75; *N. aquatica* (SC), P_n = [3.57 * ln(medium moisture)] + 10.95; *N. ogeche*, P_n = [3.43 * ln(medium moisture)] + 10.40; *N. sylvatica*, P_n = [1.63 * ln(medium moisture)] + 5.65.

CHAPTER 4. NAA IS MORE EFFECTIVE THAN IBA FOR ROOTING STEM CUTTINGS OF TWO *NYSSA* SPP.[1]

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Abstract. We conducted two experiments to determine whether *Nyssa aquatica* and *Nyssa ogeche* could be propagated from softwood cuttings. In the first experiment, two commercial auxin formulations were used, each at two concentrations. In the second study, we compared root formation among cuttings treated with 3000-ppm indole-3-butyric acid (IBA) in liquid or talc, 3000-ppm 1-naphthaleneacetic acid (NAA) in liquid, and a combination of 1500-ppm IBA and 1500-ppm NAA in liquid. Cuttings were taken from terminal and subterminal positions on juvenile stock plants and held under intermittent mist for six weeks. More terminal cuttings rooted than did subterminal cuttings. A liquid formulation of NAA or NAA and IBA was more effective than IBA at promoting root formation for both species. Up to 93% of cuttings rooted when treated with a solution containing NAA and IBA. Cuttings of *N. aquatica* treated with NAA in solution produced up to eight times more roots than cuttings treated with no auxin or IBA. For both species, three times more cuttings rooted when treated with IBA in liquid compared to IBA in powder. Results indicate *N. aquatica* and *N. ogeche* can be propagated from softwood cuttings, and rooting is enhanced by treating terminal cuttings with NAA in solution.

Index words. asexual propagation, adventitious rooting, auxin.

Species used in this study: *Nyssa aquatica* L. (water tupelo), *Nyssa ogeche* Bartr. ex Marsh. (Ogeechee tupelo).

Chemicals used in this study: indole-3-butyric acid (IBA); 1-napthalenacetic acid (NAA); Hormex® Concentrate (2400-ppm NAA, 130-ppm IBA, and 2500-ppm thiamine); Rhizopon® AA#2 (3000-ppm IBA); Rhizopon® AA#3 (8000-ppm IBA).

Significance to the Nursery Industry

Nyssa aquatica (water tupelo) and *Nyssa ogeche* (Ogeechee tupelo) are flood-tolerant trees with horticultural potential. However, their use in the horticultural industry is limited by a lack of protocols for asexual propagation that would allow for multiplication of superior genotypes. High rooting percentages (up to 93%) were achieved for both species when softwood cuttings of seedlings in their first year of growth were treated with a commercial auxin formulation of 2400-ppm NAA and 130-ppm IBA as a 60-sec dip. Cuttings taken from seedlings in their second year of growth had lower rooting percentages (maximum of 52%) than those taken from seedlings in their first year of growth. Treatment of cuttings with NAA alone at 3000 ppm or in combination with IBA (1500-ppm NAA + 1500-ppm IBA) increased rooting percentage and, for *N. aquatica*, the number of roots formed by cuttings compared to the control. Treatment of cuttings with IBA in talc did not consistently improve rooting percentage, number of roots, or root length for either species compared to the control. Cuttings treated with IBA in liquid, however, had a higher rooting percentage than those treated with IBA in talc or no auxin. We recommend treating juvenile softwood cuttings of *N. aquatica* and *N. ogeche* with NAA in liquid at 1500 to 3000 ppm. Using these methods,

genotypes that show desirable traits at the juvenile stage could be propagated asexually, but future research is needed to define protocols for propagating selections that are identified when plants are mature.

Introduction

Trees that thrive in periodically flooded soils are valuable in managed landscapes where wide fluctuations in soil moisture and aeration are common. *Nyssa aquatica* (water tupelo) and *Nyssa ogeche* (Ogeechee tupelo) are flood-tolerant species native to the southeastern United States with potential for increased use in the horticultural industry. In the wild, *N. aquatica* grows in periodically and continuously flooded soils (14). *Nyssa ogeche* grows in consistently wet soils (15) but has been cultivated successfully in drained soils (21). Thus, both species may be suited to landscapes in which large fluctuations in soil moisture occur or where saturation of soils limits plant selection.

Nyssa aquatica is fast-growing, deciduous, and can be 30 m (100 ft) tall when mature (6). Its potential uses in horticulture have not been evaluated. *Nyssa ogeche* is also deciduous but grows only 9 to 12 m (30 to 40 ft) tall. Individuals form dense, round canopies with red to burgundy fall color (6, 21). In a 13-year evaluation of 200 taxa of shade trees, *N. ogeche* was ranked as one of the best ornamentals based on growth rate, survival after transplanting, and aesthetic quality (21). However, the species remains obscure in the horticultural industry (6). Dirr (6) implies its lack of popularity is caused by inconsistent fall color and heavy crops of potentially messy fruits. Males of this polygamodioecious species could be selected to avoid problems associated with the shedding of drupes from female trees. Genotypes might also be selected for consistent fall color.

One obstacle to promoting *N. aquatica* and *N. ogeche* in the horticultural industry is a lack of protocols for asexual propagation that would permit multiplication of superior genotypes. Rooting of stem cuttings has been successful with *Nyssa sylvatica* (black gum), a closely related species; Dummer (7) reported 100% rooting of softwood cuttings taken from juvenile *N. sylvatica* and treated with indole-3-butyric acid (IBA) at 8000 ppm in talc. However, propagation of *N. aquatica* and *N. ogeche* from stem cuttings has not been documented.

Although stem cuttings of certain species form adventitious roots without exogenous auxin application, treatment with auxin may increase rooting percentage, number and length of roots formed, and uniformity of rooting (10). Indole-3-butyric acid and 1-naphthalenacetic acid (NAA) are commonly used in commercial propagation because of their consistency in promoting adventitious root formation on cuttings (10). The extent to which exogenous auxins increase rooting percentage, number of roots, and length of roots is dependent in part on species, type and concentration of auxin, carrier in which auxin is applied, and extent of wounding (10). Among other factors, adventitious root formation differs for many species according to the position from which the cutting was taken on the stock plant (17, 19), the time of the year the cutting was taken (1), age of the stock plant (4,18), and environmental factors such as irradiance (8) and temperature of the rooting medium (16).

We conducted two experiments to determine whether *N. aquatica* and *N. ogeche* could be propagated from softwood cuttings of juvenile, greenhouse-grown plants. In Expt. 1, our specific objective was to determine whether commercially available auxins could be used to increase rooting percentage and the number and length of roots on cuttings. In Expt.

2, we investigated the extent to which a liquid formulation of NAA, IBA, or a combination of both increased the same dependent variables. We also sought to determine whether IBA was more effective when in a liquid or powder. In both experiments, a final objective was to determine if the position from which the cutting was taken on the stock plant (terminal cuttings from apical positions on primary shoots vs. subterminal cuttings immediately basipetal of terminal cuttings) influenced rooting success.

Materials and Methods

Plant material. For Expts. 1 and 2, softwood cuttings of *N. aquatica* and *N. ogeche* were taken from seedlings in their first and second year of growth, respectively. Plants of *N. ogeche* were grown from seeds collected in 2006 in Levy Co., FL. Plants of *N. aquatica* were grown from seeds collected in 2006 in Levy Co., FL, Aiken Co., SC, and Union Co., IL. In Expt. 1, no provenance effects were found among cuttings of *N. aquatica* from different seed sources, so, for Expt. 2, cuttings were taken only from the plants from Aiken Co., SC. Seedlings were grown in pots with a top diameter of 15 cm (6 in) and volume of 1.8 L (112 in^3) in soilless medium (Conrad Fafard soil mix #51, Agawam, MA), irrigated daily with tap water, and fertilized once weekly with N at 250 $mg \cdot L^{-1}$ from Peters Excel All-Purpose 21N-2.2P-16.6K (Scotts, Marietta, GA). Stock plants were maintained in Ames, IA, in a glass-glazed greenhouse that was vented but otherwise was not cooled. Mean daily maximum/minimum air temperatures were 31/17C (88/63F) during the first growing season and 28/16C (82/61F) during the second growing season. No supplemental irradiance was provided. Maximum irradiance at canopy level was measured with a quantum sensor (Li-Cor, Lincoln, NE) at midday on three sunny days each year and averaged 650 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

Application of auxin treatments. Cuttings were taken from uniform stock plants on July 18, 2007 for Expt. 1 and August 2, 2008 for Expt. 2. Two cuttings with stems that were 10 cm (4 in) long were taken from each stock plant; one actively growing terminal cutting was taken, and one subterminal cutting was taken immediately below the terminal cutting. Terminal cuttings were flexible and lacked lignification. Subterminal cuttings also lacked substantial lignification but were more rigid than terminal cuttings.

For Expt. 1, two commercially available auxin formulations were used, each at two concentrations. For each species, 30 cuttings from each position (terminal or subterminal) were randomly assigned to each of five treatments: control (distilled deionized water), Rhizopon® AA #2 (3000-ppm IBA in talc) (Hortus USA Corp. Inc., New York, NY), Rhizopon® AA #3 (8000-ppm IBA in talc), full-strength Hormex® Concentrate (2400-ppm NAA, 130-ppm IBA, and 2500-ppm thiamine in water) (Brooker Chemical Corp., North Hollywood, CA), and a 1:1 (by volume) dilution of Hormex® Concentrate and distilled water. For Expt. 2, three formulations of auxin (IBA, NAA, or a combination of IBA and NAA) were applied in either liquid or powder as follows: liquid control (distilled deionized water), powder control (talc), IBA at 3000 ppm in talc (Rhizopon® AA#2), IBA at 3000 ppm in water, NAA at 3000 ppm in water, and a combination of IBA and NAA, each at 1500 ppm, in water. Ten cuttings from each position were assigned to each auxin treatment. Auxin treatments (liquid and powder) were applied to the basal 2 cm (0.8 in) of the cutting. Liquid auxin treatments were applied for 60 sec. Leaves were removed from the basal 2 cm of the stem before treating with auxin. After these leaves were removed, all cuttings had 2 to 3 fully expanded leaves remaining. Cuttings were not wounded.

Environmental conditions. Cuttings were inserted into individual 60-mL (3.7-in^3) plugs (50-cell plug trays) filled with coarse perlite and placed under intermittent mist in a glass-glazed greenhouse under 50% shade cloth with no supplemental irradiation. A dibble was used to facilitate insertion of the cuttings into the medium. Mist was controlled with an electronic leaf sensor (Phytotronics, Earth City, MO). Photosynthetically active radiation (*PAR*) was measured daily using a quantum sensor (Li-Cor, Lincoln, NE) at four locations across the bench at midday. Average *PAR* was 152 $\mu mol \cdot m^{-2} \cdot s^{-1}$ during Expt. 1 and 147 $\mu mol \cdot m^{-2} \cdot s^{-1}$ during Expt. 2. Mean daily maximum/minimum air temperatures were 31/20C (88/68F) and 29/17C (84/63F) during Expts. 1 and 2, respectively.

Data collection. Cuttings were harvested August 28, 2007 for Expt. 1 and September 13, 2008 for Expt. 2. For each cutting, the number of primary roots was recorded, and the length of the longest primary root was measured. Roots visible without magnification were counted. A cutting was considered to be rooted if at least one root was visible.

Experimental design and statistical analysis. Expt. 1 was a completely randomized design with treatments in a factorial arrangement and 30 replications, and Expt. 2 was a randomized complete block design with two blocks with five cuttings per treatment within each block. Blocks were arranged on two sides of the mist bench to account for variance in light and mist distribution. Species, cutting position, and auxin treatment were considered fixed factors. Stock plant and block were considered random factors. All data were analyzed with a MIXED model analysis of variance using SAS (v.9, SAS Inst., Cary, NC). For analyses of root length and number of roots, each species was analyzed separately, all possible interactions of fixed factors were included in the models, and only data for cuttings that rooted were analyzed. Values of root length and number of roots were log or square-root

transformed to normalize variance. For Expt. 1, analyses of rooting percentage data were performed using rooting percentages calculated for each combination of species, cutting position, and auxin treatment (percentage of 30 replicates). To obtain sufficient degrees of freedom for tests of main effects and two-way interactions, three-way interactions of rooting percentage data were not included in the models (for interactions, n = 2 to 5; for main effects, n = 4 to 10). For Expt. 2, analyses of rooting percentage data were performed the same as in Expt. 1, but rooting percentages were calculated for each combination of block, species, cutting position, and auxin treatment (for interactions, n = 4 to 12, for main effects, n = 8 to 24). Mean separations were performed using Tukey's studentized range test (HSD), and contrasts were used to compare data of interest.

Results and Discussion

Commercial auxins. Hormex® Concentrate consistently increased rooting percentage, number of roots, and root length for cuttings of both *N. aquatica* and *N. ogeche*. Rooting percentage of cuttings of *N. aquatica* and *N. ogeche* did not differ, so data presented are means of the two species. Auxin concentration did not affect rooting percentage, number of roots, or root length for cuttings of *N. aquatica* or *N. ogeche* treated with either commercial auxin. Averaged over both concentrations, 48% more subterminal cuttings rooted when treated with Hormex® Concentrate than when treated with no auxin (Table 1). Rooting percentage of terminal cuttings was not dependent on auxin treatment; across the control and all auxin treatments, 70% of terminal cuttings rooted. Cuttings of *N. aquatica* treated with Hormex® Concentrate at full-strength or as a 1:1 dilution produced eight times more roots than cuttings treated with no auxin, and the longest of these roots were twice as long as those formed without auxin (Table 2). For *N. ogeche*, treatment with Hormex® Concentrate at

either concentration resulted in twice the number of roots and a 69% increase in length of the longest root compared with the control (Table 2).

Treatment with Rhizopon® AA#2 and Rhizopon AA® #3 increased the number and length of longest roots formed on cuttings of *N. aquatica*, but not *N. ogeche*, compared with the control (Table 2). Forty-eight percent more roots were formed by cuttings treated with Rhizopon® compared with the control, and maximum root length was increased by 68% compared with the control treatment. However, the number of roots produced on cuttings of *N. aquatica* treated with Rhizopon® was one fifth of that of cuttings treated with Hormex® Concentrate. Treatment of subterminal cuttings with Rhizopon® did not increase rooting percentage compared with the control for either species (Table 1).

Hormex® Concentrate and Rhizopon® contain different formulations of auxins and are applied to cuttings in different carriers. Hormex® Concentrate contains a combination of IBA and NAA in liquid, whereas Rhizopon® contains only IBA in powder. Both the chemical form of auxin and the carrier in which it is applied can influence formation and development of adventitious roots. Cuttings of many species are more likely to root and produce larger root systems when treated with a combination of IBA and NAA than either auxin alone (5, 10), although this is not the case for all species (20). Application of auxin as a liquid rather than a powder can also increase rooting percentage or number of roots formed by cuttings (2, 11). Auxin applied in liquid is less likely to be removed from a cutting physically during insertion of the basal portion of the cutting into rooting medium compared to when auxin is applied in powder (13), and more auxin may be taken up by the cutting when applied in liquid as opposed to powder (9). For many species, however, application of auxin in talc is as effective as liquid application (12). Results from Expt. 2 indicate that both

the chemical form of auxin and the carrier in which it is applied affect rooting success of stem cuttings of *N. aquatica* and *N. ogeche*. We designed our second experiment based on the hypothesis that the larger rooting percentage, number of roots formed, and root length of cuttings treated with Hormex® Concentrate compared to Rhizopon® in Expt. 1 was a result of the presence of NAA and the liquid carrier.

Chemical form of auxin. 1-Naphthaleneacetic acid, whether applied alone or in combination with IBA, was more effective than IBA at promoting root formation on softwood cuttings of *N. aquatica* and *N. ogeche*. Cuttings treated with an auxin formulation that included NAA rooted twice as frequently as cuttings treated with IBA alone ($P = 0.0051$; Table 3). In addition, cuttings of *N. aquatica* treated with a preparation that contained NAA in liquid formed eight times more roots than cuttings treated with IBA in liquid, and cuttings treated with both NAA and IBA formed five times more roots than cuttings treated with IBA in liquid ($P = 0.0548$; Table 4). No cuttings of *N. aquatica* treated with the liquid control or IBA in talc rooted. Cuttings of *N. ogeche* treated with NAA or with both NAA and IBA had roots that were 57% longer than roots on cuttings treated with IBA in liquid ($P = 0.0171$; Table 4).

Auxin carrier. Application of IBA in liquid increased rooting percentage for cuttings of both species compared to application of IBA in talc. Three times more cuttings rooted when IBA was delivered in solution than when delivered in powder (Table 3). Treatment of cuttings with IBA in talc did not increase rooting percentages compared to the control in either experiment.

Cutting position. In Expt. 1, terminal cuttings of *N. aquatica* rooted at a greater percentage than did subterminal cuttings, but subterminal cuttings formed more roots than

did terminal cuttings. Seventy-eight percent of terminal and 58% of subterminal cuttings of *N. aquatica* rooted ($P = 0.0480$). Subterminal cuttings of *N. aquatica* produced 16 roots per cutting, whereas terminal cuttings produced 13 ($P = 0.0050$). Cutting position did not affect rooting percentage or number of roots produced by *N. ogeche* in Expt. 1. However, roots on subterminal cuttings of *N. ogeche* were longer than those on terminal cuttings; mean length of the longest root was 3.2 cm for subterminal cuttings and 2.5 cm for terminal cuttings ($P = 0.0042$). In Expt. 2, terminal cuttings of both species had a 4 percentage point higher rooting percentage than subterminal cuttings, with 27% of terminal and 23% of subterminal cuttings forming roots ($P = 0.0453$).

The low rooting percentage of subterminal cuttings compared with that of terminal cuttings observed with these *Nyssa* spp. is in contrast to several studies that reported relatively high rooting percentages with subterminal cuttings (17, 19). Differences in rooting percentage associated with cutting position may reflect biochemical gradients within the stem, variation in carbohydrate content, or differential occurrence of preformed root initials (10). It is doubtful that the success of terminal cuttings was due to a greater occurrence of preformed root initials on the cuttings we used because root initials generally form on basal portions of the stem (10). In Expt. 1, treatment of subterminal cuttings with Hormex® Concentrate resulted in a rooting percentage similar to that of terminal cuttings. Because the rooting percentage of subterminal cuttings was increased by treatment with auxin, we speculate auxin gradients within the stem limited rooting of subterminal cuttings.

Despite similar environmental conditions during both experiments, few cuttings rooted in Expt. 2 compared to Expt. 1, which limited the power to detect differences among treatments in Expt. 2. Only 5% of cuttings in the liquid control group rooted in Expt. 2,

whereas 50% rooted in Expt. 1. Similarly, 60% of cuttings treated with Rhizopon® AA #2 (IBA in talc) rooted in Expt. 1, whereas only 7% rooted in Expt. 2. Cuttings in Expt. 2 were taken two weeks later in the growing season from seedlings that were one year older than those in Expt. 1. Rooting percentage often declines with stock-plant age (3, 4), but for most species, this trend is closely tied to physiological maturation (10). Our stock plants were juvenile both times cuttings were taken. We have observed that seedlings of *N. ogeche* can become mature within three years in greenhouses, so the plants we used may have been in a transitional stage when cuttings were collected for Expt. 2. In contrast, seedlings of *N. aquatica* typically remain juvenile for several decades (14). Additional trials over multiple years with cuttings taken at different dates from stock plants of various ages would be needed to resolve temporal changes in rooting success of these species. Despite the variation over time we observed, our results demonstrate for the first time that *N. aquatica* and *N. ogeche* can be propagated asexually by using softwood cuttings from juvenile stock plants. Up to 93% of cuttings treated with auxin rooted within six weeks, which suggests that asexual propagation of specific genotypes of *N. aquatica* and *N. ogeche* could be viable in a production setting. However, further research is needed to determine whether superior genotypes could be propagated asexually from cuttings taken from mature trees. We recommend treating cuttings of both species with NAA in liquid to promote rooting.

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Table 1. Rooting percentages of subterminal cuttings of *Nyssa aquatica* and *Nyssa ogeche* in Expt. 1. Softwood cuttings were taken from juvenile, greenhouse-grown plants 10 cm (4 in) below the apex. Cuttings were treated with no auxin (liquid control), Rhizopon® AA #2 and #3, which contain 3000- and 8000-ppm indole-3-butyric acid (IBA) in talc, respectively, or Hormex® Concentrate, a liquid that contains 2400-ppm 1-naphthaleneacetic acid (NAA) and 130-ppm IBA, at full-strength or as a 1:1 dilution with distilled water. Rooting percentages for cuttings of *N. aquatica* and *N. ogeche* did not differ, so values are means of 60 cuttings, 30 per species.

Auxin treatment	Subterminal cuttings rooted (%)
Control	43b[z]
Rhizopon® AA #2	45b
Rhizopon® AA #3	55ab
Hormex® Concentrate, 1:1 dilution	88a
Hormex® Concentrate, full-strength	93a

[z]Different letters indicate differences at $P \leq 0.05$ according to Tukey's studentized range test.

Table 2. Number of primary roots and length of the longest primary root formed on stem cuttings of *Nyssa aquatica* and *Nyssa ogeche* taken from greenhouse-grown, juvenile stock plants in Expt. 1. Softwood cuttings were treated with no auxin (control) or one of two commercial auxin formulations, each at two concentrations. Rhizopon® AA #2 and #3 are talc-based and contain 3000- and 8000-ppm indole-3-butyric acid (IBA), respectively. Hormex® Concentrate, a liquid, contains 2400-ppm 1-naphthaleneacetic acid (NAA) and 130-ppm IBA. Values are means of n replicates, where n represents the number of terminal and subterminal cuttings that rooted.

Species	Auxin treatment	n	Root count (per cutting)	Longest root (cm)
Nyssa aquatica	Control	29	3c[z]	1.9c
	Rhizopon® AA #2	31	5b	3.7ab
	Rhizopon® AA #3	37	4b	2.8b
	Hormex® Concentrate, 1:1 dilution	58	23a	4.2a
	Hormex® Concentrate, full-strength	49	24a	3.6ab
Nyssa ogeche	Control	31	5c	2.1b
	Rhizopon® AA #2	41	5c	2.0b
	Rhizopon® AA #3	37	6bc	2.9ab
	Hormex® Concentrate, 1:1 dilution	47	9ab	3.5a
	Hormex® Concentrate, full-strength	41	11a	3.6 a

[z]For each species, different letters within a column indicate differences at $P \leq 0.05$ according to Tukey's studentized range test.

Table 3. Rooting percentages of terminal and subterminal cuttings of *Nyssa aquatica* and *Nyssa ogeche* in Expt. 2. Softwood cuttings were taken from juvenile, greenhouse-grown plants. Cuttings were treated with no auxin as a distilled-water or talc dip (controls), 3000-ppm indole-3-butyric acid (IBA) in talc (Rhizopon® AA #2) or water, 3000-ppm 1-naphthaleneacetic acid (NAA) in water, or a combination of 1500-ppm IBA and 1500-ppm NAA in water. Rooting percentages for cuttings of *N. aquatica* and *N. ogeche* did not differ, so values are percentages of 40 cuttings, 20 of *N. aquatica* and 20 of *N. ogeche*.

Auxin treatment	Carrier	Cuttings rooted (%)
Control	Liquid	5c[z]
Control	Powder	8bc
IBA	Powder	8c
IBA	Liquid	25ab
NAA	Liquid	50a
NAA + IBA	Liquid	52a

[z]Different letters indicate differences at $P \leq 0.05$ according to Tukey's studentized range test.

Table 4. Number of primary roots and length of the longest primary root formed on stem cuttings of *Nyssa aquatica* and *Nyssa ogeche* taken from greenhouse-grown, juvenile stock plants in Expt. 2. Softwood cuttings were treated with no auxin as a distilled-water or talc dip (controls), 3000-ppm indole-3-butyric acid (IBA) in talc (Rhizopon® AA #2) or water, 3000-ppm 1-naphthaleneacetic acid (NAA) in water, or a combination of 1500-ppm IBA and 1500-ppm NAA in water. Values are means of n replicates, where n represents the number of terminal and subterminal cuttings that rooted.

Species	Auxin treatment	Carrier	n	Root count (per cutting)	Longest root (cm)
Nyssa aquatica	Control	Liquid	0	—	—
	Control	Powder	2	3ab[z]	0.9a
	IBA	Powder	0	—	—
	IBA	Liquid	4	2b	0.5a
	NAA	Liquid	11	17a	1.2a
	NAA + IBA	Liquid	12	11ab	1.2a
Nyssa ogeche	Control	Liquid	2	3a	0.6a
	Control	Powder	1	1a	0.3a
	IBA	Powder	3	9a	0.9a
	IBA	Liquid	6	2a	0.6a
	NAA	Liquid	8	7a	1.4a
	NAA + IBA	Liquid	10	9a	1.4a

[z]For each species, different letters within a column indicate differences at $P \leq 0.05$ according to Tukey's studentized range test.

CHAPTER 5. POPULATION GENETIC STRUCTURE OF *NYSSA AQUATICA* L.

A paper to be submitted to Aquatic Botany

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Abstract. We studied the population genetics of *Nyssa aquatica* L. (water tupelo), a widespread wetland tree native to the southeastern United States, to determine if genetic structure showed evidence of a barrier to gene flow among populations east and west of the Apalachicola River, reflective of regional historical biogeography. We genotyped 157 individuals from 24 populations throughout the geographic range of *N. aquatica* by using 315 loci from three ISSR primer pairs. Analysis of molecular variance (AMOVA) and Bayesian estimates of population fixation (θ^B) revealed that moderate differentiation existed among populations, with up to 21% of the total genetic variation distributed among populations. We saw no evidence of structure indicative of isolation by distance. Evidence of regional structure reflective of historical biogeography was significant, but minimal, accounting for only 0.5% of the total variation. Evidence from Bayesian clustering analyses performed using the program STRUCTURE indicated that regional structure could be absent. Because of minimal regional differentiation, we recommend management strategies for this species focus on maintaining current levels of diversity within individual populations.

Introduction

Wetland conservation is an increasing concern as human encroachment and landscape alteration continue to cause fragmentation of wetland habitats and subsequent loss of species diversity. Recently, many studies have been aimed at describing genetic diversity and spatial genetic structure of wetland fauna and flora in order to better design conservation strategies (DeWoody et al., 2004; Jaquemyn et al., 2006; Liu et al, 2006; Rivera-Ocasio et al., 2002). For wetland plants, this research has focused mainly on threatened and endangered herbaceous annual and short-lived perennial species that have limited pollen dispersal and exist on small spatial scales (Rivera-Ocasio et al., 2002; Liu et al, 2006; Jaquemyn et al., 2006) and has emphasized two characteristic features. First, many of these plants exist in fragmented metapopulations due to habitat alteration and repeated cycles of extinction and colonization that correspond with frequent natural changes in hydrology (Lytle and Poff, 2004). Second, long-distance unidirectional seed dispersal via water can be an important component of gene flow, resulting in more genetic variation within downstream populations than within upstream populations (Akimoto et al., 1998; Gornall et al., 1998; Kudoh and Whigham, 1997; Liu et al., 2006; but see DeWoody et al., 2004).

Whereas metapopulation dynamics and unidirectional gene flow are characteristic of many studies done on small spatial and temporal scales, little is known about the genetic diversity and population genetic structure of long-lived wetland species at regional and historic scales. Fragmentation and hydrochory likely do not have the same effects on the genetic structure of widespread, long-lived, wind-pollinated species. Evidence from forest trees suggests that long-distance pollen-mediated gene flow and tree longevity may delay or even prevent differentiation and loss of genetic variation within fragmented populations at a

regional scale (Hamrick, 2004; Kramer et al., 2008). At a regional scale, the effects of unidirectional seed dispersal on patterns of nuclear genetic variation also may be overshadowed by pollen movement.

The genetic structure of widespread, long-lived, wind-pollinated trees may also be affected by regional historical biogeographic influences on range distributions, including range expansion and contraction events associated with Pleistocene glacial maxima and minima (Avise, 2000; Hewitt, 1996; Soltis et al., 1997).

Nyssa aquatica L. (water tupelo) is a large, long-lived, wind-pollinated tree that is widespread throughout floodplain and coastal forest wetlands in the southeastern United States. The species is important ecologically and economically in the region, but we know of no information describing its population genetics at a regional scale. Freshwater fauna and flora with distributions similar to that of *N. aquatica* show a predictable pattern of regional differentiation between the Gulf and Atlantic coasts, with a divide generally occurring east and west of the Apalachicola River (Avise, 2000; Lickey and Walker, 2002; Mylecraine et al., 2004). One widely accepted explanation for this separation between Atlantic and Gulf populations is that saline estuaries and salt marshes during interglacial periods created a physical barrier to gene flow at the Floridian peninsula for temperate freshwater species (Avise, 1996).

The objectives of this study were to estimate the amount of genetic variation contained within and among populations of *N. aquatica* throughout its range and to determine the extent to which population genetic structure is reflective of historic biogeography. We used inter-simple sequence repeat (ISSR) markers to assess this objective. Although ISSRs cannot be used to infer allele frequencies directly, the markers are typically

highly polymorphic and reveal spatial patterns of genetic diversity that are generally in accordance with information obtained from codominant markers (Robinson et al., 1997; Schrader and Graves, 2004a; Wolfe and Liston, 1998). Information about the amount and geographical distribution of genetic diversity within *N. aquatica* is expected to identify the primary mechanisms influencing genetic structure in this species and to provide a baseline from which regional, long-term management strategies can be developed.

Materials and Methods

Study species

Nyssa aquatica (Nyssaceae) is a diploid (2n = 22), polygamodioecious tree (*i.e.*, individual trees have staminate and perfect or pistillate and perfect flowers) native to the Lower Mississippi River Valley north to southern Illinois, the Gulf Coastal Plain from eastern Texas to western Florida, and the Atlantic Coastal Plain from southern Georgia north to southern Virginia (Figure 1) (Eyde, 1997). The floral biology and sexual structure of this species implies it is primarily outcrossing with relatively low rates of selfing. Individuals mature after approximately 30 years and can live for over 100 years (Johnson, 1990). Insects are involved in pollination, but most of the pollen is likely dispersed by wind (Eyde, 1997; Shea et al., 1993). Seeds are produced in drupes that float when mature and are primarily disseminated by water (Eyde, 1997). Seed germination requires oxygen (Shunk, 1939), so new seedlings arise only when and where water levels recede. As a consequence, seasonal changes in hydrology are crucial for the establishment of new populations of *N. aquatica*.

Sample Collection

Leaf samples were obtained from 12 individuals from each of 24 populations of *N. aquatica* distributed across its geographic range in May 2006 and preserved in silica gel

desiccant (Table 1; Figure 1). The number of populations sampled was comparable to sampling efforts in other genetic studies of trees with similar distributions (*e.g.*, Lickey and Walker, 2002; Mylecraine et al., 2004). Sample trees were spaced at least 15 m apart to avoid sampling closely related individuals, and the geographic location of each individual was recorded using a handheld global positioning system. One voucher specimen was collected from each population and submitted to the Ada Hayden Herbarium at Iowa State University (Ames, IA; Appendix 1).

DNA Extraction, Amplification, and Processing

Genomic DNA was extracted from dried leaf samples using the CTAB technique (Doyle and Doyle, 1987) at the DNA Sequencing and Synthesis Facility at Iowa State University (Ames, IA) and purified for use in ISSR-PCR using a GeneClean® Spin Kit (MP Biomedicals, Solon, OH). ISSR fragments were amplified using the fluorescent 3'-anchored primers $(CA)_6RG$, $(GTG)_3GC$, and $(CAC)_3RC$. The three primers, which were synthesized at the DNA Sequencing and Synthesis Facility at Iowa State University, were chosen based on their history of producing large numbers of reproducible bands for other woody species (Schrader and Graves, 2004a, 2004b). Reaction mixtures (total volume 25 μl) were composed of 50 ng template DNA, 1.2 μM primer, 300 μM dNTP mix (Sigma), 1x reaction buffer containing $Mg(OAc)_2$, and 1 unit KlenTaq LA DNA polymerase (Sigma). Thermocycler temperatures for PCR were: initial denaturing at 94 °C for 5 minutes, denaturing at 94 °C for 30 seconds, annealing at 47, 56, and 52 °C for primers $(CA)_6RG$, $(GTG)_3GC$, and $(CAC)_3RC$, respectively, for 45 seconds, and extension at 72 °C for 2 minutes. Thirty to 33 cycles were completed for each primer with a final extension at 72 °C

for 5 minutes. Ten percent of the samples were replicated to verify consistency of ISSR amplification.

Amplification products were genotyped at the Iowa State University DNA Sequencing and Synthesis Facility using an Applied Biosystems (ABI) Prism® 3100 Genetic Analyzer. Results were visualized using Genographer software (ver. 2.0; Banks and Benham, 2008), and ISSR bands between 100 and 500 bp-long at specific loci were scored as present (1) or absent (0). For DNA extraction, ISSR amplification, and genotyping, samples from all populations were arranged randomly in 96-well plates. Samples in which banding could not be distinguished from background noise were discarded.

Data Analyses

To examine genetic relationships among individuals and populations and to check for consistency of banding patterns among replicate samples, Nei (Lynch and Milligan, 1994) and Nei-Li distances were calculated (Nei and Li, 1979), and dendograms were constructed from distance matrices using neighbor-joining (Saitou and Nei, 1987). In the analysis of population data, bootstrap support of individual nodes for a consensus tree was calculated based on 1000 resamplings. All analyses were performed using PHYLIP software (v. 3.6; Felsenstein, 2004) except for the calculation of Nei's distance, which was performed using AFLPsurv (v.1.0; Vekemans et al., 2002).

We used AMOVA (Excoffier et al., 1992) to estimate Φ_{ST}, a multilocus analog of Wright's F_{ST} and to test for evidence of population-level and regional genetic differentiation. AMOVA analyses were performed using Euclidean pairwise distances in GenAlEx (v. 6.2; Peakall and Smouse, 2006). Two hierarchical models were tested, one model which included only population structure and one model that also took into account region (populations east

and west of the Apalachacola River). Significance of Φ_{ST} statistics were based on 999 permutations. Akaike's information criterion (AIC) was calculated using the methods in Halverson et al. (2008), and a difference of 10 or greater was considered very strong support for the model with the smaller AIC (Burnham and Anderson, 1998).

A Bayesian approach was also used to estimate the average genetic differentiation among populations, without considering regional structuring. An analog of Wright's F_{ST} (θ^B) was calculated using the program HICKORY (v. 1.1; Holsinger et al., 2002). Five runs were performed using default sampling parameters (burn-in = 50,000, sample = 250,000, thin = 50) to check consistency of the results (Tero et al., 2003). Estimates of θ^B were calculated using three separate models: a full model with noninformative priors for *f* (an analog of Wright's F_{IS}) and θ^B, an $f = 0$ model (which assumes no inbreeding), and an *f*-free model that selects values of *f* at random from the prior distribution to estimate the other parameters in the model. For each estimated parameter, a 95% credible interval was also calculated according to the methods of Holsinger et al. (2002). Deviance information criterion (DIC) values were used to select the best model, with a difference in DIC between models of greater than 6 interpreted as strong support for the model with the lower DIC value (Spiegelhalter et al., 2002).

The program STRUCTURE (v.2.3) was used to assign individuals to groups without any prior information about regional structure (Pritchard et al., 2000), which may provide a stronger test of our hypotheses about biogeographical structuring since unlike the analyses done using AMOVA, populations are not assigned a priori to regions. The program uses a Bayesian model-clustering algorithm to assign individuals to a user-defined number of K groups. Data were analyzed for K = 1 to 8 using a burn-in period of 50,000, 100,000 Markov

chain Monte Carlo (MCMC) repetitions, and assuming an admixture model. Ten iterations were run for each K to check for consistency. The most likely number of groups was chosen based on the K with the largest posterior probability of the data given K and the largest delta K (Evanno et al., 2005), a measure of the rate of change in posterior probability of the genetic data given K for successive K values. Although the delta K method correctly predicts groups for $K > 2$, the method cannot be used when $K \leq 2$.

Pairwise genetic distance [$F_{ST} / (1-F_{ST})$ and Nei's genetic distance] was plotted against straight line geographic distance for all populations and for populations within each region, and Mantel tests were used to test for a significant positive correlation between these variables as expected under isolation by distance (Mantel, 1967; Rousset, 1997). For populations in the Mississippi River Valley, correlations were also performed using riverine distance. Pairwise F_{ST} values and Nei's genetic distance were estimated using Lynch and Milligan's method (1994) in AFLPsurv (v.1.0; Vekemans et al., 2002). Mantel tests were calculated with GenAlEx (v.6.2) software using 999 permutations (Peakall and Smouse, 2006).

Results

Only 157 of the 288 individuals produced banding profiles that were strong enough to distinguish from background noise; only these individuals were included in the analyses (Table 1). Each of the three primer pairs produced over 90 reproducible bands, resulting in a total of 314 fragments ranging from 100 to 500 bp. Only 32 (10%) of these were present in all individuals, whereas 282 (90%) were polymorphic. Dendograms created using the different distance matrices based on the analyses of individual trees were similar and showed clustering of all replicate samples. Further, individuals within populations generally

clustered together; averaged over all populations, 74% of individuals from the same location clustered together. One group of populations located between a latitude of 32 and 34°N and distributed across Alabama, Georgia, and South Carolina (Populations 12, 14, 18, 21, 22, and 23) clustered together with moderate bootstrap support (74%); otherwise bootstrap support was minimal (<50%) for all nodes.

The estimate of $\Phi_{ST} = 0.21$ from AMOVA was highly significant ($P < 0.0001$) and indicates that approximately 21% of the total genetic variation was partitioned among populations, and 79% was partitioned among individuals within those populations (Table 2). Differentiation between eastern and western regions was significant ($P = 0.014$), but weak, accounting for only 0.5% of the total variation. The AIC value for the model that did not include regional differentiation was 60 points lower than that for the model that did include regional differentiation, indicating that regional structuring does little to strengthen the fit of the model. Estimates of θ^B were in accordance with results obtained from AMOVA analyses, indicating moderate differentiation among populations (Table 3). Estimates of θ^B were similar for the full and $f = 0$ models, which performed better than the *f*-free model based on DIC values (Table 3). The estimate of θ^B for the full model was 0.1463 with a 95% credible interval of 0.1360 to 0.1576 (Table 3).

Posterior probability and delta K values for the STRUCTURE analyses are presented in Table 4. Posterior probability was largest for K = 1. However, delta K values were largest for K = 4. Three of the four clusters were associated with geographical regions (Figure 2). Group 1 contained populations in the Mississippi River Valley. Group 2 contained three populations along the Gulf Coast and two populations in central and northern Alabama that are associated with the Mobile River Basin. Group 3 contained populations in the Atlantic

Coastal Plain and central Alabama. Group 4 was composed of three populations that were geographically distant: two populations from the Mississippi River Valley (Populations 1 and 7) and one population from the Atlantic Coast (Population 19). In addition, most of the individuals from two populations within the Mississippi River Valley (Populations 4 and 6) clustered with populations from the Gulf Coast/Mobile River, and individuals from Populations 12 and 14, which are located in the Mobile River Basin, clustered with populations from the Atlantic Coast. We performed another AMOVA analysis using the three geographic clusters (Groups 1 – 3) identified in this analysis. The AMOVA showed that a moderate amount of the genetic variation was partitioned among populations within each region (20%), whereas only 1% was partitioned among regions. Most of the variation (80%) was contained among individuals within populations. The AIC value for this model was similar to that obtained with the 2-region model, suggesting that neither regional model fit the data as well as the model that did not group populations regionally.

Correlations between genetic and geographic distance were minimal. Estimates of Nei's distance were slightly better correlated with log-transformed straight-line geographic distance than $F_{ST}/(1-F_{ST})$, but neither relationship was significant (Nei's distance: $r^2 = 0.082$, $P = 0.140$; $F_{ST}/(1-F_{ST})$: $r^2 = -0.038$, $P = 0.346$). Correlations between genetic and geographic (straight-line and riverine) distance within regions were also minimal and nonsignificant.

Discussion

Differentiation among populations was moderate and was similar to what has been reported for other long-lived, outcrossing perennial species that have water-aided seed dispersal. Studies using dominant markers for a variety of plants with these characteristics

have shown 25% to 28% of the total genetic variation was among populations in AMOVA analyses (Nybom and Bartish, 2000). Our data showed 21% of the variation partitioned among populations. Studies using codominant markers with bald cypress (*Taxodium distichum* L.), which has a similar range and habitat as *N. aquatica*, also revealed moderate to strong differentiation among populations (F_{ST} = 0.16) across its native range (Lickey and Walker, 2002). Higher levels of genetic differentiation have been reported for small, short-lived, selfing or insect-pollinated trees. For example, Graves and Schrader (2008) showed that at least 37% of the total genetic variation found using ISSR markers was among populations of the small, insect-pollinated tree *Dirca occidentalis* Gray in California, and Hamrick and Nason (1996) reported that species that among-population diversity can account for 51% of the total genetic diversity in selfing species.

Models including regional structure were poorly supported by the data. In AMOVA analyses, regional differentiation of populations east and west of the Apalachicola River was significant, but minimal. Additionally, although delta K values supported four clusters, posterior probability of the data supported no regional differentiation. Because the delta K method cannot be used to partition individuals into less than three clusters, the correct number of clusters for our dataset may not be correctly determined using the delta K method. Alternatively, genetic similarity may be associated with regional watersheds, possibly indicative of limited seed movement among watersheds. However, the STRUCTURE analysis for K = 4 failed to group all populations into geographically similar units, and the amount of regional differentiation was small. In addition, AIC values from AMOVA analyses suggest that the model without regional differentiation provided a much better fit to the data than if region was included in the model.

In contrast with our data, regional variation for Atlantic white cedar (*Chamaecyparis thyoides* [L.] Britton, Sterns & Poggenb.), which is also native to the Atlantic and Gulf coastal plains, accounted for up to 8% of the total genetic variation (Mylecraine et al., 2004). Atlantic and Gulf populations of Atlantic white cedar are disjunct, whereas populations of *N. aquatica* are continuous along the coasts. The negligible regional clustering among populations of *N. aquatica* and the lack of evidence of isolation by distance suggests that tree longevity and long-distance, pollen-mediated gene flow may be buffering the species against strong regional differentiation. Management strategies for *N. aquatica* should focus on preserving genetic structure at the population level.

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Table 1. Collection locations and number of genotyped samples for *N. aquatica* populations.

ID	County, State	Coordinates	# Samples
1	Union, IL	37˚21'N 89˚04'W	3
2	Stoddard, MO	36˚58'N 90˚08'W	3
3	Haywood, TN	35˚30'N 89˚13'W	5
4	Pulaski, AR	34˚35'N 92˚15'W	5
5	Ashley, AR	33˚06'N 92˚06'W	7
6	Yazoo, MS	32˚48'N 90˚37'W	8
7	Oachita, LA	32˚35'N 92˚02'W	4
8	Rapides, LA	31˚12'N 92˚34'W	3
9	Orange, TX	30˚05'N 93˚45'W	6
10	St. Tammany, LA	30˚13'N 89˚40'W	6
11	Baldwin, AL	30˚50'N 87˚54'W	5
12	Tuskaloosa, AL	33˚06'N 87˚34'W	10
13	Dallas, AL	32˚15'N 87˚07'W	5
14	Montgomery, AL	32˚25'N 85˚58'W	9
15	Limestone, AL	34˚40'N 86˚49'W	4
16	Holmes, FL	30˚48'N 85˚49'W	5
17	Dougherty, GA	31˚30'N 84˚22'W	7
18	Houston, GA	32˚24'N 83˚34'W	8
19	Long, GA	31˚41'N 81˚48'W	8

Table 1. (continued)

ID	County, State	Coordinates	# Samples
20	Bulloch, GA	32˚33’N 81˚43’W	7
21	Aiken, SC	33˚19’N 81˚52’W	11
22	Dorchester, SC	33˚15’N 80˚26’W	10
23	Florence, SC	34˚12’N 79˚33’W	8
24	Columbus, NC	34˚19’N 78˚28’W	7

Table 2. AMOVA table for models with no regional structure or regional structure between populations east and west of the Apalachicola River. Values are based on 157 individuals and 282 polymorphic loci. *P*-values were determined using 999 permutations.

Source	df	Sum of squares	Variance components	% of total variance	*P*-value
No regional structure					
Among populations	23	2058	8.700	21.0	<0.0001
Within populations	133	4367	32.841	79.0	<0.0001
Total	156	6426			
Eastern and western regions					
Among regions	1	113	0.199	0.5	0.014
Among populations within regions	22	1944	8.598	20.5	<0.0001
Within populations	133	4367	32.841	79.0	<0.0001
Total	156	6426	41.639		

Table 3. Bayesian estimates of F_{ST} (θ^B) and deviance information criterion (DIC) for each of the models used. Values are based on 157 samples, 24 populations, and 282 polymorphic loci.

Model	θ^B	DIC
Full	0.1463	15833
$f = 0$	0.1427	15836
f-free	0.1953	16026

Table 4. Posterior probability of the data for each K [Pr(X|K)] and delta K values for K = 1 – 8. Values are averaged over 10 independent runs.

K	Pr(X|K)	Delta K
1	-30352	—
2	-29760	—
3	-29208	1.97
4	-29086	4.02
5	-28968	1.12
6	-29003	0.85
7	-29045	1.03
8	-29366	0.61

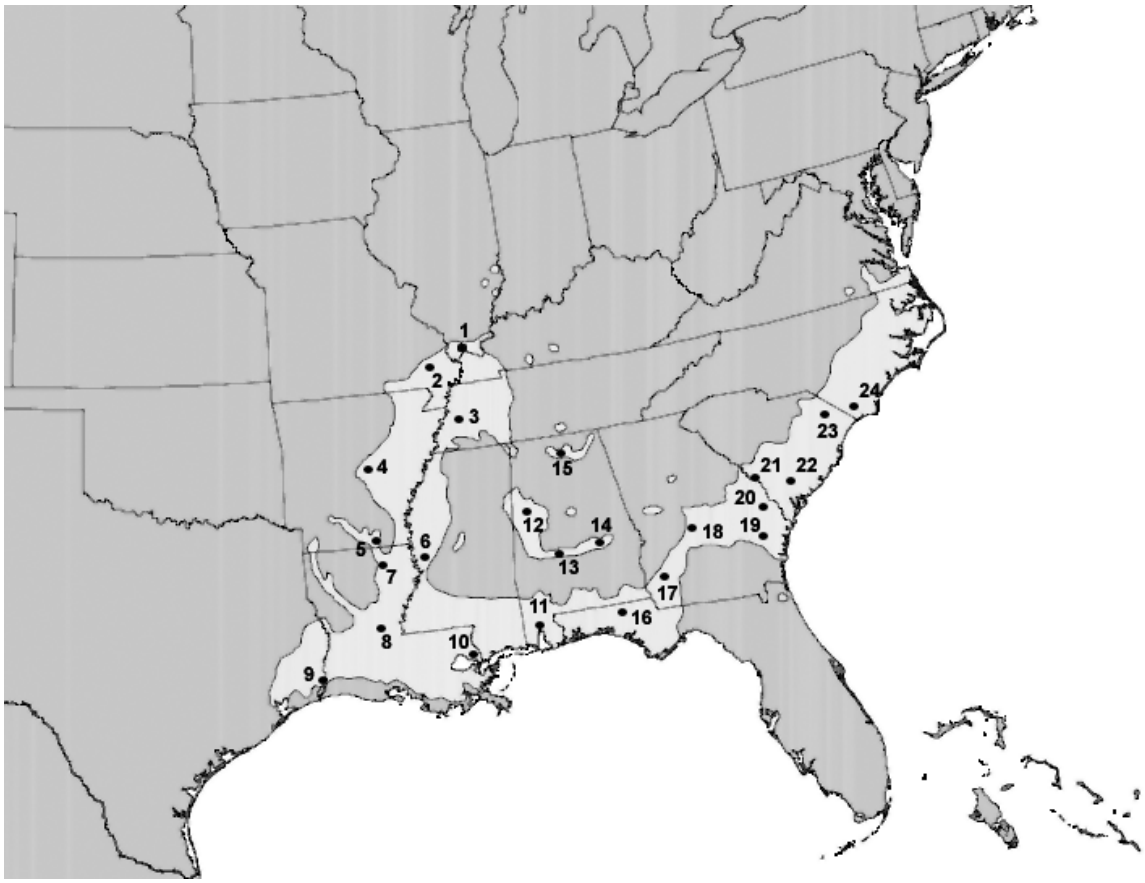

Figure 1. Locations of populations from which samples were collected. Numbers correspond to population number in Table 1. Populations 1 through 17 are west and Populations 18 through 24 are east of the Apalachicola River. Populations 1 through 11 were considered to be in the Mississippi River Valley, 12 through 17 are within the Gulf Coast / Mobile River region, and 18 through 24 are considered to be in the Atlantic Coastal Plain. The region shaded in light gray represents the current distribution of *N. aquatica*.

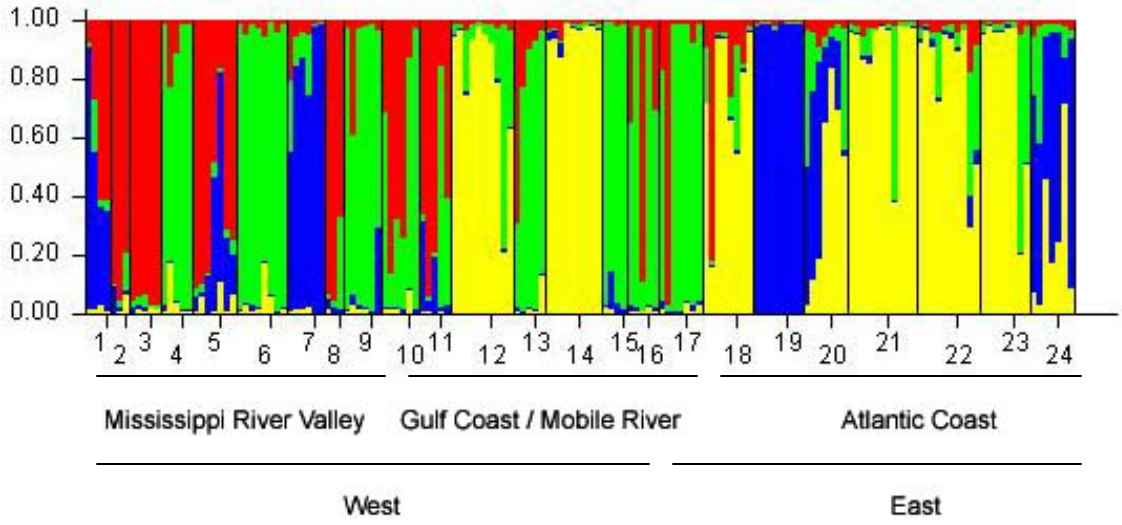

Figure 2. STRUCTURE analysis of *N. aquatica* populations for K = 4. Individuals are represented using vertical lines that are divided into colored segments representing the proportion of assignment to each of the K clusters. Populations are numbered as in Table 1, and black lines separate each population. Horizontal lines below the graphic show the geographic clusters of populations within the Mississippi River Valley, Gulf Coast / Mobile River, and Atlantic Coast as well as the populations west and east of the Apalachicola River.

CHAPTER 6. GENERAL CONCLUSIONS

Conclusions

Water management is an ongoing problem in urban landscapes. Whereas many municipalities currently face severe water shortages (Mee, 2003), others struggle with periodic flooding (Miguez et al., 2005), both of which can decrease the functional and ornamental value of horticultural plants. Plants tolerant of wide fluctuations in soil moisture are needed in urban landscapes, especially where the stresses associated with moisture extremes are intensified by soil compaction, constricted root volumes, or high temperatures (Jim, 1998; Fluckiger and Braun, 1999; Pouyat et al., 1995). These plants are likely to become more valuable in horticulture if predictions of increasing intensity of extreme hydrologic events are realized.

The wetland tree *Nyssa aquatica* was identified as a potential horticultural crop based on its survival in soils in which moisture fluctuates widely (Christensen, 1988). Although extensive research characterized the mechanisms underlying the extreme flood tolerance of this species (Eyde, 1997; Harms, 1973), and some work addressed drought tolerance (Dickson et al., 1965; Dickson and Broyer, 1972), little was known about the effects of wide fluctuations in soil moisture on the growth and physiology of *N. aquatica.*

The horticultural potential of *N. aquatica* is linked to its tolerance of extreme fluctuations in soil moisture within a single growing season. Our research justifies the consideration of *N. aquatica* for landscapes in which soil moisture fluctuates widely, but where severe or long-term water deficit is not likely. Flooding early in the season can limit the growth of young seedlings, which may benefit the trees later in the season by limiting

transpirational demand. Flood-induced decreases in above-ground growth, coupled with resistance to anoxic injury, which may be associated with quickly developed internal aeration systems in seedlings of *N. aquatica*, is one combination of traits that is likely associated with tolerance to soil moisture fluctuations.

Selections of *N. aquatica* are needed for marketing through the horticultural industry. Seedling sources rarely have traits reliable enough to market commercially, so asexual propagation methods are needed for multiplication of superior genotypes (Hartmann et al., 2002). We found that stem cuttings of juvenile plants rooted with a high rate of success (up to 93%) when treated with a solution containing 1-napthalenacetic acid (NAA) at 1500 to 3000 ppm. The high rate of rooting success in our studies and ease of multiplying plants via stem cuttings suggest that selections of *N. aquatica* could be produced easily by a commercial propagator.

We found evidence of moderate to strong differentiation among populations of *N. aquatica*, but regional structure was minimal. The life history traits of *N. aquatica* (wind-pollinated, long-lived, outcrossing) may buffer the widespread species against strong regional genetic differentiation. Selections of *N. aquatica* for horticultural use could potentially be taken from any part of the range and planted without concerns about altering regional genetic structure.

Recommendations for Future Research

Although my research provides a basis upon which *N. aquatica* can be suggested for horticultural use, specific questions about its potential uses remain unanswered. For example, extensive evaluation will be needed to determine cold hardiness and susceptibility to ornamental pests and diseases among other concerns. Plants would also need to be

evaluated for ornamental characteristics, including fall leaf color, form, and fruitlessness. One promising use for *N. aquatica*, if ornamental selections cannot be made, is as a rootstock for the ornamentally outstanding *Nyssa sylvatica*. Although we conducted preliminary studies on this topic (Appendix 2), the graft compatibility of these two species has yet to be determined.

Several of the studies presented in this dissertation used *Nyssa ogeche* as well as *N. aquatica*. Almost no literature is available on *N. ogeche*, although the species has been evaluated for ornamental potential (Williams et al., 1995). No information is available about the tolerance of this tree to environmental stresses, cold hardiness, or resistance to pests and diseases. The range of *N. ogeche* is much smaller than that of *N. aquatica*, extending through only northern Florida and southern Georgia. Within the Apalachacola River Basin, populations of *N. ogeche* have declined by 40% in the past 30 years (Darst and Light, 2008). Large decreases in population size for this relatively rare species raise concerns about conservation and genetic diversity within this species. Studies of the population genetics of the species could help determine appropriate conservation strategies.

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APPENDIX 1. HERBARIUM SPECIMENS COLLECTED FOR POPULATION GENETIC ANALYSES OF *NYSSA AQUATICA*

Ada Hayden Herbarium, Iowa State University *Nyssa aquatica* L. **Missouri, USA.** Stoddard County North side of Bluff Road between Ditch 6 and Red Mill at Mingo National Wildlife Refuge. GPS Lat: 36º 57.575' N, Long: 90º 11.576' W. Elevation: 152 m Nickolee Z. Boyer 1 9 May 2006	**Ada Hayden Herbarium, Iowa State University** *Nyssa aquatica* L. **Illinois, USA.** Union County Northeast of Mt. Olive Road along Cypress Creek at Cypress Creek National Wildlife Refuge. GPS Lat: 37º 21.072' N, Long: 89º 04.604' W. Elevation: 117 m Nickolee Z. Boyer 2 9 May 2006
Ada Hayden Herbarium, Iowa State University *Nyssa aquatica* L. **Tennessee, USA.** Haywood County Alongside Powell Road between power lines and westernmost bridge over Bear Creek at Hatchie National Wildlife Refuge. GPS Lat: 35º 30.280' N, Long: 89º 13.127' W. Elevation: 99 m Nickolee Z. Boyer 3 10 May 2006	**Ada Hayden Herbarium, Iowa State University** *Nyssa aquatica* L. **Tennessee, USA.** Haywood County Alongside Powell Road between power lines and westernmost bridge over Bear Creek at Hatchie National Wildlife Refuge. GPS Lat: 35º 30.292' N, Long: 89º 13.106' W. Elevation: 98 m Nickolee Z. Boyer 4 10 May 2006

Ada Hayden Herbarium, Iowa State University *Nyssa aquatica* L. **Missouri, USA.** Stoddard County Red Mill Pond at Mingo National Wildlife Refuge. GPS Lat: 36º 58.625' N, Long: 90º 08.032' W. Elevation: 121 m Nickolee Z. Boyer 5 10 May 2006	**Ada Hayden Herbarium, Iowa State University** *Nyssa aquatica* L. **Alabama, USA.** Limestone County South side of Greenbriar Road near Greenbriar, Alabama. Just north of Wheeler National Wildlife Refuge. GPS Lat: 34º 40.178' N, Long: 86º 49.318' W. Elevation: 178 m Nickolee Z. Boyer 6 11 May 2006
Ada Hayden Herbarium, Iowa State University *Nyssa aquatica* L. **Arkansas, USA.** Pulaski County Lorance Creek Natural Area near Bingham, Arkansas. GPS Lat: 34º 35.023' N, Long: 92º 15.119' W. Elevation: 117 m Nickolee Z. Boyer 8 12 May 2006	**Ada Hayden Herbarium, Iowa State University** *Nyssa aquatica* L. **Alabama, USA.** Tuskaloosa County Just off Maxwell Loop Road, immediately west of Englewood, Alabama. GPS Lat: 33º 06.856 N, Long: 87º 34.814' W. Elevation: 52 m Nickolee Z. Boyer 7 11 May 2006
Ada Hayden Herbarium, Iowa State University *Nyssa aquatica* L. **Mississippi, USA.** Grenada County Growing in Tippo Bayou on the northeast side of Tallahatchie National Wildlife Refuge northwest of Shaw Road. GPS Lat: 33º 47.214' N, Long: 90º 08.143' W. Elevation: 43 m Nickolee Z. Boyer 9 12 May 2006	**Ada Hayden Herbarium, Iowa State University** *Nyssa aquatica* L. **Mississippi, USA.** Grenada County Growing in Tippo Bayou on the northeast side of Tallahatchie National Wildlife Refuge northwest of Shaw Road. GPS Lat: 33º 47.198' N, Long: 90º 08.146' W. Elevation: 43 m Nickolee Z. Boyer 10 12 May 2006

Ada Hayden Herbarium, Iowa State University *Nyssa aquatica* L. **Louisiana, USA.** Oachita Parish Northeastern finger of Black Bayou Lake. GPS Lat: 32º 35.671' N, Long: 92º 02.790' W. Elevation: 27 m Nickolee Z. Boyer 11 13 May 2006	**Ada Hayden Herbarium, Iowa State University** *Nyssa aquatica* L. **Louisiana, USA.** Oachita Parish Northeastern finger of Black Bayou Lake. GPS Lat: 32º 35.618' N, Long: 92º 02.731' W. Elevation: 28 m Nickolee Z. Boyer 12 13 May 2006
Ada Hayden Herbarium, Iowa State University *Nyssa aquatica* L. **Mississippi, USA.** Yazoo County West of West Levee Road near the south end of Panther Swamp National Wildlife Refuge. GPS Lat: 32º 48.354' N, Long: 90º 37.262' W. Elevation: 44 m Nickolee Z. Boyer 13 13 May 2006	**Ada Hayden Herbarium, Iowa State University** *Nyssa aquatica* L. **Arkansas, USA.** Ashley County Growing in Wildcat Lake at Felsenthal National Wildlife Refuge. GPS Lat: 33º 04.795' N, Long: 92º 06.077' W. Elevation: 21 m Nickolee Z. Boyer 14 15 May 2006
Ada Hayden Herbarium, Iowa State University *Nyssa aquatica* L. **Louisiana, USA.** Rapides Parish Growing along a small stream northeast of the 273-287 junction in Kisatchie National Forest, Calcasieu District. GPS Lat: 31º 12.301' N, Long: 92º 34.627' W. Elevation: 22 m Nickolee Z. Boyer 15 16 May 2006	**Ada Hayden Herbarium, Iowa State University** *Nyssa aquatica* L. **Texas, USA.** Orange County Growing along Adams Bayou at Shangri La Botanical Gardens and Nature Center in Orange, Texas. GPS Lat: 30º 32.931' N, Long: 93º 47.861' W. Elevation: 24 m Nickolee Z. Boyer 16 16 May 2006

Ada Hayden Herbarium, Iowa State University *Nyssa aquatica* L. **Alabama, USA.** Montgomery County Just north of I-85 on the west side of Atlanta Highway near Waugh, Alabama. GPS Lat: 32º 24.756' N, Long: 85º 57.821' W. Elevation: 59 m Nickolee Z. Boyer 20 20 May 2006	**Ada Hayden Herbarium, Iowa State University** *Nyssa aquatica* L. **Louisiana, USA.** St. Tammany Parish White Kitchen at the junction of Hwy 90 and Hwy 190. GPS Lat: 30º 13.720' N, Long: 89º 40.984' W. Elevation: 6 m Nickolee Z. Boyer 18 18 May 2006
Ada Hayden Herbarium, Iowa State University *Nyssa aquatica* L. **Georgia, USA.** Dougherty County North side of Hwy 62 between Pretoria and Holt, Georgia. GPS Lat: 31º 30.230' N, Long: 84º 22.790' W. Elevation: 37 m Nickolee Z. Boyer 22 23 May 2006	**Ada Hayden Herbarium, Iowa State University** *Nyssa aquatica* L. **South Carolina, USA.** Aiken County Southwest side of Silver Bluff Audubon Sanctuary near Jackson, South Carolina. GPS Lat: 33º 19.292' N, Long: 81º 52.258' W. Elevation: 35 m Nickolee Z. Boyer 26 24 May 2006
Ada Hayden Herbarium, Iowa State University *Nyssa aquatica* L. **Georgia, USA.** Long County North side of Hwy 84 along the edge of Patterson Swamp between Doctortown and Ludowici, Georgia. GPS Lat: 31º 41.000' N, Long: 81º 48.141' W. Elevation: 9 m Nickolee Z. Boyer 24 24 May 2006	**Ada Hayden Herbarium, Iowa State University** *Nyssa aquatica* L. **North Carolina, USA.** Lenoir County Albrittons Landing on the north side of Hwy 55 just east of the Neuse River. GPS Lat: 35º 17.561' N, Long: 77º 29.333' W. Elevation: 13 m Nickolee Z. Boyer 28 25 May 2006

Ada Hayden Herbarium, Iowa State University

Nyssa aquatica L.

Georgia, USA.
Bulloch County
Growing on the banks of the Ogeechee River on the west side of Hwy 301 near Dover, Georgia.

GPS Lat: 32º 33.675' N, Long: 81º 43.072' W.
Elevation: 60 m

Nickolee Z. Boyer 25 24 May 2006

Ada Hayden Herbarium, Iowa State University

Nyssa aquatica L.

South Carolina, USA.
Florence County
Pee Dee River Swamp north of Hwy 76/301 near the boat launch.

GPS Lat: 34º 12.185' N, Long: 79º 33.480' W.
Elevation: 20 m

Nickolee Z. Boyer 30 25 May 2006

Ada Hayden Herbarium, Iowa State University

Nyssa aquatica L.

Virginia, USA.
Isle of Wight County
South side of Hwy 460 east of Zuni, Virginia.

GPS Lat: 36º 50.890' N, Long: 76º 48.298' W.
Elevation: -2 m

Nickolee Z. Boyer 32 30 May 2006

Ada Hayden Herbarium, Iowa State University

Nyssa aquatica L.

North Carolina, USA.
Bertie County
Growing on the banks of the Cashie River just south of Windsor, North Carolina.

GPS Lat: 35º 59.048' N, Long: 76º 56.445' W.
Elevation: 0 m

Nickolee Z. Boyer 31 26 May 2006

Ada Hayden Herbarium, Iowa State University

Nyssa aquatica L.

North Carolina, USA.
Columbus County
South side of Hwy 214 between Lake Waccamaw and Bolton, North Carolina. .

GPS Lat: 34º 19.313' N, Long: 78º 28.088' W.
Elevation: 22 m

Nickolee Z. Boyer 29 25 May 2006

APPENDIX 2. POTENTIAL OF *NYSSA AQUATICA* L. (WATER TUPELO) AS A ROOTSTOCK FOR *NYSSA SYLVATICA* MARSH. (BLACK GUM)

Introduction

Nyssa aquatica L. (water tupelo) is a North American tree native to periodically and continually flooded wetlands that can survive many years of inundation (Hook, 1984) but is also tolerant of short periods of water deficit (Dickson et al., 1965; Dickson and Broyer, 1972) and wide fluctuations in soil moisture (see Chapter 2). Because of its tolerance to extremes in soil moisture, *N. aquatica* is an attractive option for managed landscapes in which soil moisture and oxygen fluctuate widely. However, *N. aquatica* lacks the outstanding fall color and graceful pyramidal form of other ornamental *Nyssa* species, such as *Nyssa sylvatica* Marsh. (black gum) and *Nyssa sinensis* Oliv. (Chinese tupelo), which may reduce the marketability of this tree in ornamental horticulture.

One potential use for *N. aquatica* is as a rootstock for *N. sylvatica*, a popular ornamental that is also native to North America. *Nyssa sylvatica* is tolerant of some waterlogging, but growth of seedlings is reduced in flooded soils (Nash and Graves, 1993; Hook, 1984). Use of *N. aquatica* as a rootstock could potentially increase the range of moisture and soil oxygen conditions into which *N. sylvatica* could be planted in the landscape. In addition, grafting could potentially improve vigor of the slow-growing species and reduce the time needed to achieve saleable size (Flemer, 1989).

Selections of *N. sylvatica* are budded or grafted onto seedling rootstock in the nursery industry (Dirr, 1998; Dummer, 1968). Successful grafts of *N. sinensis* on *N. sylvatica* stock have also been reported (Lancaster, 1987). For plants within a species or genus, grafting can

be successful for some combinations and unsuccessful for others, but grafts of more closely related plants are typically more successful (Hartmann et al., 2002). Graft success is also influenced by timing, technique, environmental conditions, and pathogens, among other things (Hartmann et al., 2002).

The mechanisms underlying graft incompatibility are not well understood (Pina and Errea, 2005; Hartmann et al., 2002; Santamour, 1988). Moreover, graft compatibility can be difficult to ascertain for woody plants because visual signs of incompatibility can appear many years after functional vascular tissue is produced (Hartmann et al., 2002). Thus, much research has been aimed at finding methods of detecting graft incompatibility for woody plants. One promising method for assessing graft compatibility was developed for *Prunus* and involves micrografting undifferentiated callus tissue in vitro. Grafted callus sections are then visualized microscopically for evidence of metabolic dysfunctions at the graft union (Errea et al., 2001). This method offers several advantages over other methods of detecting incompatibility, including quick detection of possible incompatibility once callus is established, avoidance of potential contamination, and tight control of environmental conditions.

We used two methods to test graft compatibility of seedlings of *N. sylvatica* and *N. aquatica*. First, we conducted a series of tests in which seedlings of both species were budded or grafted in the greenhouse. Second, we attempted to assess compatibility using micrograft techniques in vitro.

Materials and Methods

Greenhouse trials

Seedlings of *N. aquatica* used for rootstock and scions were grown from seeds collected in 2006 in Levy Co., FL. Seedlings were germinated in the spring of 2007 and grown in 1.8-L pots with a top diameter of 15.24 cm in soilless medium (Conrad Fafard soil mix #51, Agwam, MA). Seedlings of *N. sylvatica* used in the 2008 trials were obtained in 2007 from a commercial nursery (Urban Forestry Services, Micanopy, FL) and were from a Florida seed source. The 1-year-old liners were transplanted into 1.8-L pots in soilless medium (Conrad Fafard soil mix #51, Agwam, MA) in the spring of 2007. Seedlings of *N. sylvatica* used in the 2009 trial were germinated in 2006 from seeds collected at the Minnesota Landscape Arboretum (Chanhassen, MN) from an open-pollinated tree that arboretum records show originated from the wild in Lincoln Co., MO. Seedlings were grown in 2.5-L square containers (10-cm diameter, 25-cm tall) in media composed of composted bark, peat, and coarse sand in an outdoor nursery at the Minnesota Landscape Arboretum until June 2007 when they were moved to Iowa State University Research Greenhouses (Ames, IA) and transplanted into 2.8-L pots in soilless medium (Conrad Fafard mix #51, Agawam, MA). During the growing season, all seedlings for all trials were watered daily with tap water and fertilized once weekly with 250 mg $N \cdot L^{-1}$ from Peters Excel All-Purpose 21N-2.2P-16.6K (Scotts, Marietta, GA). All seedlings were maintained in a glass-glazed greenhouse that was vented but otherwise was not cooled, and no supplemental irradiation was provided. Greenhouse temperature was kept between 0 and 20 C in the winter and between 10 and 32 C during the growing season, and maximum PAR was 650 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

Grafting trials began on 26 January 2008, 2 February 2008, 1 March 2008, and 14 February 2009. For each trial, four graft combinations were used: *N. sylvatica* on *N. sylvatica* rootstock (autograft), *N. aquatica* on *N. aquatica* rootstock (autograft), *N. aquatica* on *N. sylvatica* rootstock, *N. sylvatica* on *N. aquatica* rootstock. Rootstocks were cut at 8 to 10 cm above the medium surface, and scions were 12 cm long. After placing stock and scion together, grafts were wrapped with parafilm. All seedlings were dormant when grafting was performed. Grafted plants were arranged in a completely randomized design on greenhouse benches and were watered weekly with tap water.

In January and February 2008, whip grafts of three and 10 replicates, respectively, of each combination were prepared. In March 2008, ten replicates of each combination were grafted, five of which were saddle grafts and five of which were whip grafts. All grafts were placed under a tent made of clear polyethylene sheeting and placed in a greenhouse until May 2008. Temperature averaged 15 $\pm$ 3 C (11/32 min/max).

In 2009, five replicates of each combination were whip grafted. Scions and rootstocks were dipped in fungicide solution (Daconil, 3.4 $mL \cdot L^{-1}$) to prevent fungal growth. Grafts were placed in glass-glazed greenhouse without supplemental irradiation until April 2009. Mean daily maximum/minimum temperature was 17/22 C.

In vitro grafting

Two-cm-long stem sections were taken from new growth of seedlings of *N. aquatica* and *N. sylvatica*. Seedlings of both species were from Florida seed sources as described above. Sections were surface sterilized for 10 minutes in 20% hypochlorite solution, rinsed three times in sterile distilled water, and recut into 1-cm sections. Individual stem sections were then placed into 190-ml glass jars (6 cm diameter) containing a modified woody plant

medium (Lloyd and McCown, 1980) supplemented with 2% sucrose, 0.6% Difco-Bacto agar, 4.5 µ*M* 2,4-dichlorophenoxyacetic acid, and 0.0003 *M* ascorbic acid (pH = 5.3). Jars were moved to a growth chamber where temperature was maintained under a 16-hour photoperiod at 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$ with incandescent lights at 25 C. Stem sections were transferred every three weeks.

After six weeks in subculture, 4-cm by 4-cm pieces of callus were cut from stem sections and placed side by side in modified woody plant medium as described above. Callus tissue was solid, but felt spongy rather than dense. There were six replications of each of the four graft combinations described for the grafting trials. Grafts were held together with sterile cable ties (2.2-cm diameter; Gardner Bender, Milwaukee, WI) that were placed around the two graft partners. Micrografts were arranged in a growth chamber in a completely randomized design and incubated under the same conditions as the stem sections. Grafts were transferred after three weeks and examined after five weeks.

Results

None of the grafts conducted in the greenhouse in 2008 or 2009 were successful. In 2008, fungal growth was noted on 72% of the grafts, and only 10% formed callus. One autograft of *N. aquatica* from the February 2008 trial survived and put on new growth through the spring but was dead by July of that year. Callus was noted on 50% of the grafts from the 2009 trial. Callus was more prolific on *N. aquatica* scions and rootstocks than on *N. sylvatica*, but none of the combinations formed strong graft unions. By April 2009, all scions were dead.

Grafts in vitro were also unsuccessful. Although callus tissue was uncontaminated and alive at the time the experiment concluded, no graft unions had formed, and the graft interface for most samples was discolored.

Discussion

The failure of grafts in the control group (*N. sylvatica* autografts) indicates that factors other than incompatibility were influencing graft success in all of our trials. In 2008, fungal contamination was widespread. Stock plants were watered often, and continually moist soil prior to grafting probably contributed to the pathogen problems. In addition, polyethylene tents that were designed to increase humidity around newly grafted plants restricted airflow. Callus was more prolific in 2009 when rootstocks and scions were treated with fungicide and grafts were not placed under polyethylene sheeting. However, even then, no successful grafts formed.

Dummer (1968) reported that grafts of *N. sylvatica* do best if they are not watered after grafting. The plants in our trials were watered regularly because they were growing in small pots. Giving plants less water may have improved graft success by slowing the initial burst of growth and allowing stronger graft unions to form.

Micrografts of callus tissue were also unsuccessful. Fugii and Nito (1972) reported that callus type influenced strength of the graft union for callus grafts of fruit trees. Callus with loosely packed cells formed weak graft unions, while callus with a dense cellular structure formed stronger graft unions. We used 2,4-D to induce quick formation of callus (Errea et al., 2001), and although callus from individual stem sections was selected for compactness, all callus was somewhat spongy. Hand sections of fresh tissue indicated that air space was abundant in callus produced by stem sections of *N. aquatica*. This type of

callus may have prevented strong graft unions from forming in vitro. Culturing callus using medium supplemented with indole-3-butyric acid (IBA) promoted formation of dense callus for fruit trees (Fugii and Nito, 1972). A similar formulation may be better for obtaining callus for grafting *Nyssa* species as well.

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